Mineralocorticoids decrease the activity of the apical small-conductance K channel in the cortical collecting duct

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Mineralocorticoids decrease the activity of the apical small-conductance K channel in the cortical collecting duct. Am J Physiol Renal Physiol 289: F1065–F1071, 2005; doi:10.1152/ajprenal.00063.2005.—We used the patch-clamp technique to examine the effect of DOCA treatment (2 mg/kg) on the apical small-conductance K (SK) channels, epithelial Na channels (ENaC), and the basolateral 18-pS K channels in the cortical collecting duct (CCD). Treatment of rats with DOCA for 6 days significantly decreased the plasma K from 3.8 to 3.1 meq/l and reduced the activity of the SK channel, defined as NP, from 1.3 in the CCD of control rats to 0.6. In contrast, DOCA treatment significantly increased ENaC activity from 0.01 to 0.53 and the basolateral 18-pS K channel activity from 0.67 to 1.63. Moreover, Western blot analysis revealed that DOCA treatment significantly increased the expression of the nonreceptor type of protein tyrosine kinase (PTK), c-Src, and the tyrosine phosphorylation of ROMK in the renal cortex and outer medulla. The possibility that decreases in plasma K induced by mineralocorticoids may result from increases in the electrochemical driving force for K secretion rather than stimulation of apical K conductance is tested in the present study by investigating the effect of DOCA on the activity of SK channels in the CCD of the rat kidney.

METHODS

Preparation of CCDs. Pathogen-free Sprague-Dawley rats of both sexes (age 6 wk) were used in the experiments. The animals were purchased from Taconic Farms (Germantown, NY) and placed on a normal rat chow (1.1% wt/wt). The animals were divided into the experimental group that received DOCA injection (2 mg/kg body wt) for 2, 4, and 6 days and the control group that received oil injection. In a separate set of experiments, rats were kept on a high-K (HK) diet (10%) and received DOCA injection for 6 days. Rats were killed by cervical dislocation, and kidneys were removed immediately. Several thin slices of the kidney (<1 mm) were cut and placed on the ice-cold Ringer solution until dissection. The dissection was carried out at room temperature, and two watchmaker forceps were used to isolate the single CCD. To immobilize the tubules, they were placed onto a 5 × 5-mm coverglass coated with polylysine (Sigma, St. Louis, MO). The coverglass was transferred to a chamber (1,000 μl) mounted on an inverted Nikon microscope. The CCDs were superfused with HEPES-buffered NaCl solution, and the temperature of the chamber was maintained at 37 ± 1°C by circulating warm water surrounding the chamber. The method to patch the basolateral membrane of the CCD has been described previously (26).

Patch-clamp technique. An Axon200A patch-clamp amplifier was used to record channel current. The K current and the Na current were low-pass filtered at 1 KHz and at 50–100 Hz, respectively, using an eight pole Bessel filter (902LPF, Frequency Devices, Haverhill, MA). Data were digitized by Axon interface 1200 and stored on the hard-drive of an IBM-compatible Pentium computer (Gateway 2000).

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We used the pClamp software system 6.04 (Axon Instruments, Burlingame, CA) to analyze the data. Channel activity was defined as $N_{P_o}$, and was calculated from data samples of 60-s duration in the steady state as follows

$$N_{P_o} = \sum (t_1 + t_2 + \ldots + t_i)$$

where $t_i$ is the fractional open time spent at each of the observed current levels.

**Immunoprecipitation and Western blot analysis.**

Renal cortex and outer medulla were dissected and homogenized as described previously. The ROMK antibody was added to the protein samples (500 μg) harvested from renal cortex and outer medulla with a ratio of (4 μg/1 mg protein). The mixture was gently rotated at 4°C overnight, followed by incubation with 25 μl of protein A/G plus agarose (Santa Cruz Biotechnology) for an additional 2 h at 4°C. The tube containing the mixture was centrifuged at 3,000 rpm and the agarose bead pellet was mixed with 25 μl of SDS sample buffer containing 4% SDS, 100 mM Tris-HCl (pH 6.8), 20% glycerol, 200 mM dithiothreitol, 0.2% bromophenol blue. After the sample was boiled for 5 min, we loaded the supernatant to separate the proteins by electrophoresis on 10% SDS-polyacrylamide gels and transferred them to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) and rinsed and washed with 0.05% Tween 20-TBS buffer. We used ECL (Amersham Pharmacia Biotech) to detect the protein bands and the intensity of the corresponding band.

**Fig. 1.** Bar graph showing the channel activity defined as $N_{P_o}$ of the SK channels in the cortical collecting duct (CCD) from control and the DOCA-treated rats. Experiments were performed in cell-attached patches. *Significant difference ($P < 0.05$).

**Fig. 2.** A: bar graph showing the channel activity of epithelial Na channel (ENaC) in the CCD from control and the DOCA-treated rats. Experiments were carried out in cell-attached patches. *Significant difference ($P < 0.05$). B: channel recording demonstrating ENaC activity in the CCD from control and DOCA-treated rats. The channel closed level is indicated by C or a dotted line. The experiments were performed in cell-attached patch and pipette holding potential was −40 mV (hyperpolarization).

**Fig. 3.** A: channel recording showing the activity of the basolateral K channels in the CCD from control and the DOCA-treated (6 days) rats. The channel-closed line is indicated by C and dotted lines. The trace indicated by * is extended to show the fast time resolution. The experiments were performed in cell-attached patches, and the holding potential was 0 mV. B: bar graph showing the channel activity of the basolateral 18-pS K channels in the CCD from control and the DOCA-treated rats. *Significant difference ($P < 0.01$).
was determined with Alpha DigiDoc 1000 (Alpha Innotech, San Leandro, CA). The monoclonal tyrosine phosphorylation antibody (PY20 or 4G10) was purchased from Upstate. For studying the expression of c-Src, protein samples extracted from renal cortex and outer medulla were separated by 8% SDS-polyacrylamide gels. The monoclonal c-Src antibody was purchased from Transduction Laboratories (Lexington, KY).

**Experimental solution and statistics.** The pipette solution contained (in mM) 140 KCl, 1.8 MgCl₂, and 10 HEPES (pH 7.4) for studying K channel activity and 140 mM NaCl and 1.8 MgCl₂ and 10 HEPES (pH 7.4) for studying ENaC. The bath solution for cell-attached patches was composed of (in mM) 140 NaCl, 5 KCl, 1.8 CaCl₂, 1.8 MgCl₂, and 10 HEPES (pH 7.4). Data are shown as means ± SE, and the paired Student’s t-test was used to calculate the significance between the control and experimental groups. Statistical significance was taken as \( P < 0.05. \)

**RESULTS**

We first examined the activity of the apical SK channels in the CCD from control rats and animals treated with DOCA (200 \( \mu \)g/100 g) for 2, 4, and 6 days. The data summarized in Fig. 1 show that channel activity defined by \( N_{Po} \) in the CCD was 1.3 ± 0.2 (n = 24) under control conditions and decreased significantly to 0.6 ± 0.2 (n = 22) from rats treated with DOCA for 6 days. We also examined the effect of DOCA treatment on ENaC activity in the CCD and confirmed that the activity of ENaC in the CCD in rats on a normal rat chow (0.5% Na) was almost absent and \( N_{Po} \) was only 0.01 ± 0.07 (n = 14). DOCA treatment significantly increased Na channel activity from 0.01 ± 0.07 to 0.42 ± 0.10 (2 days, n = 11), 0.53 ± 0.10 (4 days, n = 11), and 0.49 ± 0.10 (6 days, n = 10), respectively (Fig. 2A). Figure 2B is a typical recording showing the activity of ENaC in the CCD from rats treated with DOCA for 0, 2, and 6 days, respectively.

After determining that DOCA increased the apical Na channel activity but decreased apical SK channel activity, we extended the study by examining the activity of basolateral K channels in the CCD from rats treated with DOCA for 6 days. Figure 3A is a recording demonstrating the activity of the basolateral 18-pS K channel in the CCD from control rats and from animals treated with DOCA for 6 days. From inspection of Fig. 3A, it is apparent that the activity of the basolateral 18-pS K channel was significantly higher in the CCD treated with DOCA than that observed in control animals. Also, we noticed that the amplitude of the basolateral K channel was larger in the CCD of the DOCA-treated rats than that of control animals at the same pipette holding potentials. This is presumably due to the hypopolarization of basolateral membrane in the CCD. Figure 3B summarizes results obtained from 12 patches showing that DOCA treatment increased the \( N_{Po} \) from

**Table 1. Effect of DOCA on plasma Na, Cl, and K**

<table>
<thead>
<tr>
<th></th>
<th>Na, mM</th>
<th>K, mM</th>
<th>Cl, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>140±1.5</td>
<td>3.80±0.08</td>
<td>94±1.0</td>
</tr>
<tr>
<td>DOCA</td>
<td>138±2.4</td>
<td>3.11±0.2*</td>
<td>96±1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. Plasma Na, K, and Cl concentrations in control and DOCA (6 day)-treated rats (n = 5). *Significant difference between control and the DOCA-treated group.
control value 0.67 ± 0.11 to 1.62 ± 0.14. Therefore, DOCA treatment significantly stimulated the basolateral K channel activity and apical ENaC activity but suppressed the apical SK channel activity.

An increase in ENaC activity induced by DOCA was expected to stimulate Na absorption and raise the electrochemical driving force for K across the apical membrane. This should enhance the K secretion and decrease the plasma K concentration (hypokalemia). This speculation was confirmed by measuring plasma K concentrations under control conditions and in DOCA-treated rats (Table 1). DOCA treatment did not alter plasma Na or Cl concentrations but significantly decreased the plasma K concentration from the control value, 3.80 ± 0.08 to 3.11 ± 0.2 meq (n = 5). Thus application of mineralocorticoids enhanced K secretion and led to decreases in plasma K concentrations.

We previously demonstrated that low-K intake stimulates the expression of PTK (28, 29) and increases the tyrosine phosphorylation of ROMK (11) which enhances the internalization of SK channels in the CCD (12, 24). Thus we tested the hypothesis that decreases in apical SK channel activity following DOCA treatment were also due to the stimulation of PTK activity and tyrosine phosphorylation of ROMK channels. We used Western blot analysis to examine the expression of c-Src, a nonreceptor type of PTK expressed in the CCD, as a representative member of Src family PTK (12). Figure 4A is a typical Western blot showing that the expression of c-Src in the renal cortex and outer medulla was 85 ± 10% higher in rats receiving DOCA for 6 days than that in control animals (n = 4). DOCA treatment increased not only c-Src expression but also tyrosine phosphorylation of ROMK. Figure 4B is a typical Western blot demonstrating that DOCA treatment increased progressively the tyrosine phosphorylation of ROMK (n = 3). Although DOCA treatment had no effect on control value 0.67 ± 0.11 to 1.62 ± 0.14. Therefore, DOCA treatment significantly stimulated the basolateral K channel activity and apical ENaC activity but suppressed the apical SK channel activity.

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Fig. 5. A: Western blot showing the expression of c-Src in the renal cortex and outer medulla in rats on normal (1.1%)-K (NK) and high-K (HK; 10%) diets and the DOCA-treated rats on HK diet, respectively. B: channel activity in the CCD from control rats (NK) and the DOCA-treated rats on a HK diet.

Fig. 6. A: channel recording demonstrating the effect of herbimycin A (1 μM) on the apical SK channels in a cell-attached patch. The channel closed level is indicated by C. Top: time course of the experiment and 3 parts of the trace indicated by numbers are extended to show the fast time resolution. B: effect of DOCA and DOCA+herbimycin A on the apical SK channel activity. The experiment was performed in a cell-attached patch. *Significant difference from the control value.
ROMK expression, tyrosine phosphorylation of ROMK in rats treated with DOCA for 6 days was 90 ± 10% higher than that of control rats.

To test the hypothesis that decreases in plasma K rather than DOCA are responsible for the DOCA-induced stimulation of Src-family PTK and decreasing SK channel activity, we examined the effect of DOCA on c-Src expression in the kidney from rats on a HK diet. Figure 5A is a Western blot showing that the expression of c-Src significantly decreases in renal cortex and outer medulla from rat on a HK diet (50 ± 5%) and DOCA-treated rats on a HK diet (40 ± 5%; n = 3) compared with the control animals. Moreover, the patch-clamp experiments have also demonstrated that channel activity (1.8 ± 0.4; n = 7) in the CCD from DOCA-treated rats on a HK diet is higher than that of control rats (1.19 ± 0.5), although the difference is not significant (Fig. 5B).

The notion that increases in PTK activity induced by DOCA treatment were responsible for suppression of apical SK channel activity in the CCD from rats receiving DOCA for 6 days was supported by experiments in which inhibition of PTK increased the activity of the apical SK channels. Figure 6A is a channel recording showing the effect of herbinycin A (1 μM) on the apical SK channels in the CCD from a DOCA-treated rat. It is clear that addition of herbimycin A increased channel activity. Figure 6B summarizes the effect of herbimycin A on apical SK channel activity in the CCD from rats treated with DOCA for 6 days, showing that inhibition of PTK increased NPo from 0.5 ± 0.1 to 2.1 ± 0.2 (n = 13). Thus inhibition of PTK increases the activity of ROMK-like SK channel activity in the CCD.

**DISCUSSION**

The main finding of the present study is that mineralocorticid treatment decreased apical SK channel activity in rats on a normal-K diet. The similar observation has been reported by Palmer’s study in which infusion of aldosterone decreased SK channel activity. The similar observation has been reported by Palmer’s study in which infusion of aldosterone decreased SK channel activity. Figure 5A is a Western blot showing that the expression of c-Src significantly decreases in renal cortex and outer medulla from rat on a HK diet (50 ± 5%) and DOCA-treated rats on a HK diet (40 ± 5%; n = 3) compared with the control animals. Moreover, the patch-clamp experiments have also demonstrated that channel activity (1.8 ± 0.4; n = 7) in the CCD from DOCA-treated rats on a HK diet is higher than that of control rats (1.19 ± 0.5), although the difference is not significant (Fig. 5B).

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The present study demonstrates that DOCA-induced stimulation of the renal K secretion is not the result of increases in ROMK-like SK channel activity. The observation that DOCA treatment significantly increases the activity of ENaC and the basolateral 18-pS K channels supports the notion that increases in the electrochemical driving force play an important role in mediating the DOCA-induced stimulation of renal K secretion. Stimulation of basolateral K channels leads to hyperpolarization of cell membrane potential which not only increases the driving force for Na entry but also decreases the driving force.
for K recycling across the basolateral membrane. Accordingly, K entering the cells via Na-K-ATPase would be secreted into the lumen to keep the intracellular K content constant. It has been reported that 9–16 days of DOCA treatment hyperpolarized the cell membrane potential such that it exceeded the K-equilibrium potential (20). Accordingly, basolateral K channels can serve as a route for K entering the cell across the basolateral membrane. In addition to 18-pS K channels, an intermediate-conductance and a large-conductance K channel are also in the basolateral membrane (8). Although we did not examine their activity, it is possible that both intermediate- and large-conductance K channels are also activated by DOCA treatment.

Increases in K secretion lower plasma K and cause hypokalemia. Thus DOCA treatment-induced decreases in ROMK-like SK channel activity act as a compensatory mechanism to prevent excessive K loss. Three lines of evidence indicate that DOCA treatment-induced decrease in SK channel activity was the result of activation of PTK-dependent signaling: 1) DOCA treatment stimulated c-Src expression and tyrosine phosphorylation of ROMK in the CCD from rats on a control K diet; 2) inhibition of PTK significantly increased the SK channel activity; and 3) DOCA did not decrease SK channel activity in the CCD from rats on a HK diet in which c-Src expression decreased. The effect of DOCA on c-Src expression is most likely the result of decreases in plasma K rather than a direct effect on PTK. This speculation was supported by two lines of evidence: 1) the expression of c-Src decreased in DOCA-treated rats on a HK diet; and 2) a significant increase in c-Src expression was observed in the kidney from rats treated with DOCA for more than 4 days. This is also consistent with the finding that there was no significant difference in plasma K in rats treated with DOCA for fewer than 4 days (Wei Y, Babiniona E, Sterling H, Jin Y, and Wang W-H, unpublished observations).

It has been shown that SGK stimulates the phosphorylation of ROMK and enhances the insertion of the ROMK channel into the plasma membrane in X. laevis oocytes (33). Moreover, deletion of SGK has been shown to impair the renal K secretion in response to HK intake (10). Because the expression of SGK increased in response to aldosterone (25), it is expected that aldosterone should increase the apical SK channel activity. However, infusion of aldosterone in rats has been shown to increase ENaC activity but have no effect on ROMK channel activity (16). Also, we observed that DOCA treatment did not increase the SK channel activity in the CCD from rats treated with DOCA for 2 days, whereas the expression of c-Src in the kidney from rats treated with DOCA for 2 days was not significantly different from the control value, it is possible that DOCA treatment may stimulate the activity of PTK. It is also possible that the mechanism by which mineralocorticoids stimulate ROMK insertion in the cell model may be suppressed by a PTK-dependent signaling pathway. However, such a mechanism may be demonstrated in rats fed with a HK diet because a HK diet suppresses PTK expression.

Figure 7 is a model of principal cells illustrating a possible mechanism by which DOCA regulates the activity of the apical SK channel, ENaC, and basolateral 18-pS K channel. DOCA treatment stimulates the basolateral K channels and ENaC and increases the electrochemical gradient for K secretion which leads to an enhanced K secretion in the collecting duct. This leads to decreases in plasma K concentrations (hypokalemia). As a consequence, Src family PTK expression increases and tyrosine phosphorylation of ROMK channels is enhanced. Stimulation of tyrosine phosphorylation of ROMK facilitates the internalization of apical SK channels which would help in maintaining excessive K waste.

We conclude that mineralocorticoids increase ENaC and basolateral K channel activity but decrease apical SK channel activity by stimulation of tyrosine phosphorylation of ROMK channels.

GRANTS

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