Na channel expression and activity in the medullary collecting duct of rat kidney

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Frindt G, Ergonul Z, Palmer LG. Na channel expression and activity in the medullary collecting duct of rat kidney. Am J Physiol Renal Physiol 292: F1190–F1196, 2007. First published January 2, 2007; doi:10.1152/ajprenal.00399.2006.—The expression and activity of epithelial Na+ channels (ENaC) in the medullary collecting duct of the rat kidney were examined using a combination of whole cell patch-clamp measurements of amiloride-sensitive currents (I\textsubscript{Na}) in split-open tubules and Western blot analysis of α-, β-, and γ-ENaC proteins. In the outer medullary collecting duct, amiloride-sensitive currents were undetectable in principal cells from control animals but were robust when rats were treated with aldosterone (I\textsubscript{Na} = 960 ± 160 pA/cell) or fed a low-Na diet (I\textsubscript{Na} = 440 ± 120 pA/cell). In both cases, the currents were similar to those measured in principal cells of the cortical collecting duct from the same animals. In the inner medullary collecting duct, currents were much lower, averaging 120 ± 20 pA/cell in aldosterone-treated rats. Immunoblots showed that all three ENaC subunits were expressed in the cortex, outer medulla, and inner medulla of the rat kidney. When rats were fed a low-Na diet for 1 wk, similar changes in α- and γ-ENaC occurred in all three regions of the kidney; the amounts of full-length as well as putative cleaved α-ENaC protein increased, and the fraction of γ-ENaC protein in the cleaved state increased at the expense of the full-length protein. The appearance of a presumably fully glycosylated form of β-ENaC in Na-depleted animals was observed mainly in the outer and inner medulla. These findings suggest that the capability of hormone-regulated, channel-mediated Na reabsorption by the nephron extends at least into the outer medullary collecting duct.

ENaC; aldosterone; outer medulla; inner medulla

AMILORIDE-SENSITIVE EPITHELIAL Na channels (ENaC) mediate reabsorption of Na\textsuperscript{+} is the distal part of the nephron (6, 9). Their function has been studied most directly using the patch clamp mainly in the cortical collecting duct (CCD) (16–18) and more recently in the connecting tubule (CNT) (5). Their expression and role in transport in the medullary collecting duct are less clear. Immunocytochemical assays have detected ENaC protein in the outer segment [outer medullary collecting duct (OMCD)] but staining of the inner segment [inner medullary collecting duct (IMCD)] was absent or weak (2, 11). An amiloride-sensitive conductance in the apical membrane of rabbit OMCD cells was demonstrated using microelectrode recordings (12). However, no active transport of Na\textsuperscript{+} could be detected in unidirectional flux measurements in isolated, perfused OMCD segments (25, 26). In the isolated, perfused IMCD, transepithelial potentials were very low and small effects of amiloride on apical membrane voltage (24) and net Na\textsuperscript{+} flux (20) were observed, suggesting that electrogenic Na\textsuperscript{+} transport through channels is minimal. However, quite substantial inhibition of net Na fluxes were measured in vivo using microcatheterization or split-droplet approaches (23, 27). In this paper, we use electrophysiological techniques and immunoblotting to investigate the presence of apical Na\textsuperscript{+} channels in the medullary collecting duct and their regulation by mineralocorticoids.

METHODS

Animals. All animal-handling procedures were approved by the Institutional Animal Care and Use Committee of Weill Medical College. Sprague-Dawley rats of either gender (150–170 g), raised free of viral infections (Charles River Laboratories, Kingston, NY), were fed sodium-deficient rat diet (MP Biomedicals, Solon, OH) or a modified diet that matches the low-Na diet but that contains 1% NaCl (MP Biomedicals) for 6–8 days. Some animals were fed the 1% NaCl diet and implanted subcutaneously with osmotic minipumps (model 2002, Alza, Palo Alto, CA) for 6–7 days to increase levels of circulating aldosterone. Aldosterone was dissolved in polyethylene-glycol 300 at 2 mg/ml to give a calculated infusion rate of 1 μg/h.

Two or three thin coronal slices were made near the hilum of the kidney for dissection of collecting ducts. Using fine forceps, the cortical segment was isolated from medullary rays. The outer medullary duct was dissected after removing the cortex from the slice. Increasingly smaller bundles of tubules were separated with forceps from the inner through the outer medulla until a segment of collecting duct became partially free. This was cleaned of attached tubule segments. For the inner medullary duct, the field of dissection was illuminated from underneath the dissection dish and short pieces of ducts were isolated from thin loop limbs. All segments of collecting ducts were split open with a thin needle and forceps and transferred to a coverslip coated with Cell Tak (BD Biosciences, Bedford, MA) for electrophysiological measurements.

In some cases, the rats were given drinking water with 3% sucrose, which was consumed avidly, for 18 h to reduce the medullary osmolarity and the consequent osmotic shock to the cells upon exposure to isotonic dissection solution. This preparation did not noticeably alter either the appearance of the tubules or the experimental results.

Electrophysiology. Measurement of amiloride-sensitive currents in principal cells of the collecting duct followed procedures described previously (4, 5).

For whole cell clamp measurements, tubules were superfused with solutions prewarmed to 37°C containing (in mM) 135 Na methanesulfonate, 5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 2 glucose, 5 mM BaCl\textsubscript{2}, and 10 HEPES adjusted to pH 7.4 with NaOH. The patch-clamp pipettes were filled with solutions containing (in mM) 7 KCl, 123 aspartic acid, 20 CsOH, 20 TEAOH, 5 EGTA, 10 HEPES, 3 MgATP, and 0.3 NaGDPβS with the pH adjusted to 7.4 with KOH. The total concentration of K\textsuperscript{+} was ~120 mM. Pipettes were pulled from hematocrit tubing, coated with Sylgard, and fire polished with a microforge. Pipette resistances ranged from 2 to 5 MΩ. Amiloride-sensitive currents were measured as the difference in current with and without 10 μM amiloride in the bath solution.

Semi quantitative immunoblotting. Polyclonal antibodies against the α-, β-, and γ-subunits of the rat ENaC were based on short peptide...
sequences in the NH₂ terminus of α-ENaC (amino acids 46–68), and the COOH termini of β-ENaC (amino acids 617–638) and γ-ENaC (amino acids 629–650), as described previously (3, 14). Antisera were purified using peptide-linked agarose bead affinity columns (Sulfolink Kit, Pierce Biotechnology). The basic characterization of these antisera was published previously (3).

To compare ENaC protein abundance between groups of rats, semiquantitative immunoblotting was carried out as described previously (3, 14, 15). Briefly, whole kidneys were dissected into cortex, outer medulla, and inner medulla and homogenized with a glass homogenizer (Wheaton, Millville, NJ) in ice-cold isolation solution containing 250 mM sucrose/10 mM triethanolamine buffer, pH 7.4, with 1 μg/ml leupeptin and 0.1 mg/ml phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). The homogenate was centrifuged at 1,000 g for 10 min to sediment unbroken cells and nuclei. The supernatant was processed for immunodetection. Total protein was measured (BCA Kit, Pierce Biotechnology, Rockford, IL). Equal amounts of protein (40–50 μg/sample) were solubilized at 70°C for 10 min in Laemmli sample buffer and were resolved on 4–12% bis-Tris gels (Invitrogen, Carlsbad, CA) by SDS-PAGE. For immunoblotting, the proteins were transferred electrophoretically from unstained gels to PVDF membranes. After being blocked with BSA, membranes were incubated overnight at 4°C with primary antibodies against α-, β-, or γ-subunits at 1:500 or 1:1,000 dilutions. Anti-rabbit IgG conjugated with alkaline phosphatase was used as a secondary antibody. The sites of antibody-antigen reaction were visualized with a chemiluminescence substrate (Western Breeze, Invitrogen) before exposure to X-ray film (Biomax ML, Kodak, Rochester, NY). Band densities were quantitated using a Quantity One densitometer and acquisition system (Bio-Rad Laboratories, Hercules, CA).

**Modeling.** We extended a previously developed mathematical model of Na and K transport by the CCD (7) to the OMCD. We used values of apical Na permeability (PNa) determined from measurements of ISa made in the OMCD as described previously (7). The interstitium Na concentration was assumed to be 280 mM, or twice that in the cortex. Other membrane parameters were assumed to be similar to those of the CCD, including apical K⁺ conductance (440 nS/mm tubule), basolateral K⁺ conductance (5,800 nS/mm), and maximal basolateral Na/K pump activity (47.6 nA/mm). The largest uncertainty in applying this model, particularly in the conditions of low luminal Na⁺, was the value of the paracellular conductance. This parameter was therefore allowed to vary.

**RESULTS**

I-V plots for whole cell currents in the presence and absence of amiloride are shown in Fig. 1 for principal cells of the CCD, OMCD, and IMCD. In all cases, the nephron segments were isolated from animals treated with aldosterone for 7 days to maximize channel activity. Both the qualitative and quantitative aspects of the amiloride-sensitive currents (ISa) were similar in the CCD and OMCD. In the IMCD, the magnitude of the ISa was well below those reported above for the OMCD, and also much smaller than those measured in the CCDs of the same animals. Because of the small size of the amiloride-sensitive currents even under presumably optimal conditions, and because of the relatively high technical difficulty of preparing the tubules for patch-clamp measurements, we did not further investigate the channel activity in IMCD cells.

Figure 2 summarizes the mean values of ISa in CCD vs. OMCD and IMCD cells. For both OMCD and IMCD values are plotted together with those measured in CCDs isolated from the same animals. The mean CCD currents were significantly different in the two sets of experiments. These findings indicate that there was no significant difference in Na channel activity in CCD and OMCD. However, currents in the IMCD were only ~20% as large as those in the CCDs of the same animals.

We also examined OMCD cells from animals on a normal diet. Similar to cells of the CCD, no effect of amiloride could be detected under these conditions (Fig. 3A), and ISa was not significantly different from zero. Thus Na channel activity, at
least as measured in vitro, is dependent on elevation of plasma aldosterone.

Levels of circulating aldosterone can also be increased in a more physiological way by feeding animals a low-Na diet. When rats were Na-deprived for 1 wk, a significant $I_{\text{Na}}$ was detected in cells from the OMCD (Fig. 3B). Again, these currents were similar to those of the CCD from the same kidneys. The absolute values of $I_{\text{Na}}$ were lower in these Na-depleted rats than in the animals treated with aldosterone. However, these animals were studied at different times and the results are not strictly comparable. Mean values of $I_{\text{Na}}$ in CCDs and OMCDs from these sets of measurements are shown in Fig. 4.

Previous studies indicated both quantitative and qualitative effects of Na depletion and mineralocorticoid infusion on ENaC proteins from whole rat kidneys (3, 14, 15). There is no information available on the comparative effects of these maneuvers on the abundance of ENaC in different parts of the kidney. We therefore carried out immunoblots of homogenates of rat kidney after dissecting the organ into cortex, outer medulla, and inner medulla, comparing animals under control conditions with those on a low-Na diet for 1 wk. The results, shown in Figs. 5, 6, and 7, indicate that Na depletion has similar effects on ENaC subunits from the three regions. As reported previously for whole kidney, the antibody to the $\alpha$-ENaC subunit recognized several different peptides. Two of these, at $\sim$85 and 30 kDa, were seen in all three regions of the kidney, and both of these were increased in abundance during Na depletion. We previously interpreted these as representing the full-length form and a proteolytic fragment of $\alpha$-ENaC, respectively (3). A third band of $\sim$60 kDa apparent molecular mass which was also reported previously had a lower abundance and was not consistently affected by Na depletion; we do not know whether this band is a form of ENaC or whether it represents nonspecific staining. Although it has an apparent molecular mass similar to that expected of the cleaved $\alpha$-ENaC, the antibody directed against the NH$_2$-terminal should not recognize that fragment. The $\beta$-ENaC subunit appeared as a presumably full-length species of 85–95 kDa in all three regions. In the outer and inner medulla, the abundance of this peptide was increased in the Na-depleted animals relative to controls and some of the protein migrated at an increased apparent molecular mass. In a previous study using whole kidney homogenates, we showed that this reflected an increase in the endoglycosidase H-resistant glycosylation of the $\beta$-subunit in response to the low-Na diet (3). This effect was not apparent in the cortex. Two forms of the $\gamma$-ENaC subunit were observed, presumably corresponding to the full-length and proteolytically cleaved peptides (3). In all regions of the kidney, we found a decrease in the full-length, 85-kDa form and an increase in the smaller 70-kDa form with the low-Na diet. In the cortex, both high and low molecular mass species appeared as multiple discrete bands. The significance of this pattern is unclear.

To quantitatively evaluate the possible contributions of Na channels in the collecting duct, we first estimated the maximal transport rates that could be achieved. We used the maximal $I_{\text{Na}}$/cell measured in aldosterone-treated animals at an optimal electrical driving force of $\sim$80 mV across the apical membrane and an estimate of 510 cells/mm tubule (5). This gave active transport rates of 3.1 pmol·s$^{-1}$·mm$^{-1}$ for the CCD, 4.1

Fig. 2. Summary of $I_{\text{Na}}$ measured at $-100$ mV for cells from rats treated with aldosterone for 1 wk. Data represent means ± SE for 13 cells to 19 cells from the CCD, OMCD, and IMCD similar to those in Fig. 1. The 2 groups of CCD data come from the same animals as the measurements from matched OMCDs and IMCDs, respectively. *Significantly different from matched CCD values.

Fig. 3. Amiloride-sensitive whole cell currents in principal cells of the OMCD in control rats fed a diet with a normal salt content (A) and in rats fed a low-Na diet for 1 wk (B). Currents measured in the absence (■) and presence (●) of 10 μM amiloride are plotted together with the difference currents (●) representing $I_{\text{Na}}$.
pmol·s⁻¹·mm⁻¹ for the OMCD, and 0.5 pmol·s⁻¹·mm⁻¹ for the IMCD. While these may reflect unidirectional fluxes that could be achieved by in vitro perfused tubules, net transport will also depend on other factors, in particular the transepithelial resistance. Furthermore, transport in vivo will depend on the delivery of Na to the tubule and on the luminal K concentration and apical K permeability.

Rather than attempt a complete reconstruction of transport along the collecting duct, we focused on the simpler question of what is the lowest Na concentration that can be maintained in the tubules using the Na channels as the main transport system. For this purpose, we adapted a mathematical model of the rat CCD developed previously (7). The minimal Na concentration that can be maintained will depend on the rate of active transport, determined in large part by the activity of apical Na channels, and on the rate of leak into the lumen through the paracellular pathway. Figure 8 shows the minimal concentration in the OMCD, assuming maximal Na channel activity, as a function of the paracellular conductance (Gpar). To keep Na⁺ at 1 mM, the paracellular conductance must be at most 0.12 mS/cm². The minimal Na⁺ depended on the apical K conductance as well, as illustrated in Fig. 8A. A high conductance decreased the minimal Na⁺ by hyperpolarizing the apical membrane and increasing the driving force for Na entry through the channels. This effect was most pronounced at higher values of Gpar; the results with the tighter epithelia were affected much less. Increasing the concentration of K⁺ in the lumen increased the minimal Na⁺ concentration by depolarizing the apical membrane (Fig. 8B). Variations in the other model parameters, such as the maximal pump rate, the pump kinetics, and the basolateral K conductance, had relatively minor effects.

DISCUSSION

The existence and/or importance of ENaC-mediated Na transport in the medullary collecting duct has not been clearly established. In isolated, perfused rabbit OMCDs isolated from the outer stripe of the medulla, Koeppen (12) reported that amiloride depolarized the transepithelial potential and hyperpolarized the cell potential, measured with intracellular microelectrodes, consistent with block of apical Na channels. However, Stokes (25, 26) did not detect active transport of Na from unidirectional fluxes, although diffusional transport across the epithelium could be measured. This paper presents the first direct measurements of Na⁺ channel activity in freshly isolated medullary collecting ducts. The most surprising finding was the high level of Na⁺ channel activity, assessed as amiloride-sensitive current, in cells from the OMCD. In fact, there was no detectable difference in the activities of the OMCDs and the CCDs of the same animals. Our data suggest that as far as channel-mediated Na transport is concerned, the OMCD can be considered as an extension of the CCD, at least in the rat. It is possible that the difference with the transport measurements in rabbit OMCD reflects species variation.

Net Na flux was observed in the isolated, perfused rat IMCD (20). However, only a small fraction of this transport was inhibited by amiloride. Consistent with that finding, Stanton...
observed significant but modest effects of the drug on transepithelial and cell potentials in isolated, perfused tubules impaled with microelectrodes. In both of these studies, the transepithelial voltage was small, at most a few millivolts, implying that the electrogenic transport processes such as those mediated by Na channels may play a minor role, or that the shunt conductance was very high. On the other hand, in vivo micropuncture of rat IMCD indicated substantial transport of Na\(^{+}/H^+\), particularly when the animals were Na deprived (1). Similarly, microcatheterization of the IMCD indicated high rates of Na reabsorption that were largely blocked by amiloride (23). Furthermore, micropuncture measurements of isotonic fluid transport in split droplets implied rates of Na transport in the IMCD in vivo that were comparable to those in the proximal tubule and were also largely amiloride inhibitable (27). In addition, cells isolated from the IMCD and studied in suspension displayed an amiloride-sensitive component of oxygen consumption (29).

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We evaluated whether the measured activity of Na channels could account for Na concentrations of <1 mM in the urine in conditions of a low-Na diet. We assumed that most of the filtered Na is reabsorbed by upstream segments and that the major role of the collecting duct produced and maintained the final reduction in concentration. Figure 8 shows that the OMCD is able to carry out this task using the Na channels but only if the transepithelial resistance is >8 kΩ·cm\(^2\). The ability of the Na channel-dependent transport system in the collecting duct to reduce luminal Na\(^+\) is also affected by both the luminal K\(^+\) concentration and the apical K\(^+\) conductance. In the OMCD, the luminal K\(^+\) concentration is usually high due to secretion by upstream segments and reabsorption of water. Furthermore, the K\(^+\) conductance may be considerably lower than in the cortex (12, 13). Both of these factors will make a low urine Na\(^+\) concentration more difficult to achieve. The situation is even worse in the IMCD, where the measured Na\(^+\) channel and predicted channel-mediated transport are much lower, and the backflux of Na\(^+\) into the tubular lumen may be larger due to the high Na\(^+\) concentration in the interstitium.

The kidney needs to regulate Na\(^+\) and K\(^+\) excretion separately, despite the fact that transport rates for the two ions can
and to accomplish the final regulation of Na as the CNT and CCD, known to express apical K channels could be used for this purpose, as has been postulated with channel activation during Na depletion (3, 4, 14, 15). This change in glycosylation may explain why this phenomenon was not observed in previous studies (3). This heterogeneity does not appear to be essential for channel activation as it was not observed in the cortex, where upregulation of the channels is well documented, and was also not seen with aldosterone treatment, which mimics the effects of Na-depletion on ENaC protein (3). Both the cause of this change in β-ENaC and its physiological significance remain unclear.

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REFERENCES