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### Research Article

The terminal nephron segment, the inner medullary collecting duct (IMCD), absorbs Na<sup>+</sup> by an electrogenic process that involves the entry through an apical (luminal) membrane Na<sup>+</sup> channel. To understand the nature of this Na<sup>+</sup> channel, we employed the patch clamp technique on the apical membrane of primary cultures of rat IMCD cells grown on permeable supports. We found that all ion channels detected in the cell-attached configuration were highly selective for Na<sup>+</sup> (Li<sup>+</sup>) over K<sup>+</sup>. The open/closed transitions showed slow kinetics, had a slope conductance of 6-11 pS, and were sensitive to amiloride and benzamil. Nonselective cation channels with a higher conductance (25-30 pS), known to be present in IMCD cells, were not detected in the cell-attached configuration, but were readily detected in excised patches. The highly selective channels had properties similar to the recently described rat epithelial Na<sup>+</sup> channel complex, rENaC. We therefore asked whether rENaC mRNA was present in the IMCD. We detected mRNA for all three rENaC subunits in rat renal papilla and also in primary cultures of the IMCD. Either glucocorticoid hormone or mineralocorticoid hormone increased the amount of alpha-rENaC subunit mRNA but had no effect on the mRNA level of the beta-rENaC or gamma-rENaC subunits. From these data, taken in the context of other studies on the characteristics of Na<sup>+</sup> selective channels and the distribution [...]

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# rENaC Is the Predominant Na<sup>+</sup> Channel in the Apical Membrane of the Rat Renal Inner Medullary Collecting Duct

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## Abstract

The terminal nephron segment, the inner medullary collecting duct (IMCD), absorbs Na<sup>+</sup> by an electrogenic process that involves the entry through an apical (luminal) membrane Na<sup>+</sup> channel. To understand the nature of this Na<sup>+</sup> channel, we employed the patch clamp technique on the apical membrane of primary cultures of rat IMCD cells grown on permeable supports. We found that all ion channels detected in the cell-attached configuration were highly selective for Na<sup>+</sup> (Li<sup>+</sup>) over K<sup>+</sup>. The open/closed transitions showed slow kinetics, had a slope conductance of 6–11 pS, and were sensitive to amiloride and benzamil. Nonselective cation channels with a higher conductance (25–30 pS), known to be present in IMCD cells, were not detected in the cell-attached configuration, but were readily detected in excised patches. The highly selective channels had properties similar to the recently described rat epithelial Na<sup>+</sup> channel complex, rENaC. We therefore asked whether rENaC mRNA was present in the IMCD. We detected mRNA for all three rENaC subunits in rat renal papilla and also in primary cultures of the IMCD. Either glucocorticoid hormone or mineralocorticoid hormone increased the amount of  $\alpha$ -rENaC subunit mRNA but had no effect on the mRNA level of the  $\beta$ -rENaC or  $\gamma$ -rENaC subunits. From these data, taken in the context of other studies on the characteristics of Na<sup>+</sup> selective channels and the distribution of rENaC mRNA, we conclude that steroid stimulated Na<sup>+</sup> absorption by the IMCD is mediated primarily by Na<sup>+</sup> channels having properties of the rENaC subunit complex. (*J. Clin. Invest.* 1995. 96:2748–2757.) Key words: patch clamp • ion channel • aldosterone • subunits • apical membrane.

## Introduction

As the final structure capable of altering the composition of the urine, the renal inner medullary collecting duct (IMCD)<sup>1</sup> plays

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1. Abbreviations used in this paper: IMCD, inner medullary collecting duct; CCD, cortical collecting duct; I<sub>sc</sub>, short-circuit current; MC, mineralocorticoid; GC, glucocorticoid; GAPDH, glyceraldehydephosphate dehydrogenase.

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an important role in the final regulation of Na<sup>+</sup> excretion and thus overall Na<sup>+</sup> homeostasis. The major rate limiting step in overall transepithelial Na<sup>+</sup> transport by the IMCD is the entry of Na<sup>+</sup> across the apical (luminal) membrane through a Na<sup>+</sup> channel. Steroid hormones such as aldosterone enhance Na<sup>+</sup> transport through this channel (1–3).

The nature of the Na<sup>+</sup> channel residing in the apical membrane of the IMCD is not clear. There are at least three classes of epithelial Na<sup>+</sup> channels based on their single channel characteristics (4, 5). One class is highly selective for Na<sup>+</sup> over K<sup>+</sup> and has a single channel conductance of 5–10 pS. Another type does not select for Na<sup>+</sup> over K<sup>+</sup> and has a single channel conductance of 25–30 pS. A third type has intermediate characteristics. All three types can be inhibited by amiloride (4).

Some investigators have proposed that the predominant Na<sup>+</sup> channel is nonselective to cations (6–8). Three features support this contention. First, nonselective cation channels are present in excised patches of cultured rat IMCD cells. Second, they can be inhibited by amiloride (7, 9), a characteristic of Na<sup>+</sup> transport across the apical membrane of both cultured and native IMCD (1, 10). Third, the channels' activity can be inhibited by cGMP (8). This feature makes the nonselective cation channel an attractive target for inhibiting transepithelial Na<sup>+</sup> transport by atrial natriuretic peptide (6, 11–14).

However, other investigators have proposed that the nonselective cation channel may not be the major pathway for Na<sup>+</sup> absorption by the IMCD, at least under normal conditions. First, the nonselective nature of the channel should permit some degree of K<sup>+</sup> secretion, but under normal conditions K<sup>+</sup> secretion is minimal or absent (1, 15, 16). Second, electrogenic (i.e., ion channel mediated) Na<sup>+</sup> transport is confined to the distal nephron segments such as the collecting duct. However, nonselective cation channels can be detected in most nephron segments (17). Third, the regulation of the nonselective cation channel in the IMCD suggests that it is not active under normal physiologic conditions. Activity in an excised patch requires a cytoplasmic [Ca<sup>2+</sup>] of 1  $\mu$ M or greater (9, 18), a concentration that is larger than would normally be found in most cells. Furthermore, its activity is inhibited by physiologic concentrations of ATP (9, 18).

Recently, several investigators have expressed Na<sup>+</sup> channel activity in oocytes (19–25) and have cloned 3 cDNA molecules from the rat ( $\alpha$ -,  $\beta$ -, and  $\gamma$ -rENaC) and human that appear to be integral parts of a highly selective Na<sup>+</sup> ion channel. Na<sup>+</sup> channels produced by expressing these three rENaC subunits in oocytes (20) have single channel characteristics similar to Na<sup>+</sup> selective channels from the renal cortical collecting duct (CCD) and cell models thereof (5, 26–30). They are highly selective for Na<sup>+</sup> (and Li<sup>+</sup>) over K<sup>+</sup>, remain open or closed for relatively long periods of time (often more than 500 ms), and have single channel conductances of 4–10 pS (depending on the conditions of measurement).

Three lines of evidence indicate that the rENaC complex represents an important target for steroid hormones such as aldosterone. First, Na<sup>+</sup> channels from apical membranes of aldosterone stimulated epithelial cells have single channel characteristics similar to the expressed rENaC complex (20, 26, 29, 31); aldosterone increases the number of these channels (29, 31). Second, the known target tissues for steroid stimulated Na<sup>+</sup> transport (descending colon, lung, and CCD) contain mRNA for all three subunits (20, 24, 25, 32). Third, steroid hormones added to cell culture models increase the abundance of (at least) the  $\alpha$  subunit mRNA (33, 34), and a low Na<sup>+</sup> diet, given to animals to increase circulating aldosterone, increases (at least)  $\gamma$ -rENaC mRNA in the colon (22).

To test the hypothesis that rENaC is the predominant Na<sup>+</sup> channel in the IMCD, we addressed three questions. (a) Does the apical membrane of IMCD cells contain highly selective Na<sup>+</sup> channels? (b) Does the inner medulla express mRNA for the rENaC subunits? (c) Does glucocorticoid or mineralocorticoid hormone increase the abundance of rENaC mRNA in IMCD cells?

## Methods

**Cell culture.** Primary cultures of Wistar rat (Harlan Sprague Dawley, Inc. Indianapolis, IN) IMCD cells were prepared using the hypotonic lysis method as described previously (1, 2, 35, 36). Rats were anesthetized with methoxyflurane before decapitation. The kidneys were removed and the inner medulla was dissected, minced, and incubated at 37°C in an isotonic solution containing 0.1% collagenase (Boehringer-Mannheim, Indianapolis, IN) for 2–3 h. Cells were then exposed to a hypotonic solution and subsequently recovered by low speed centrifugation.

Cells to be used for patch clamp experiments were seeded onto collagen-treated Transwell-clear® filter bottom cups (Costar Corp., Cambridge, MA). Cells for RNA isolation were grown on collagen-treated 30 mm diameter Millicell PCF® filter bottom cups (Bedford, MA). In addition, cells were seeded on 13-mm Millicell PCF® filter bottom cups for transepithelial electrical measurements (1, 2, 35, 36) to be certain that the monolayers were confluent and that the steroids increased the short-circuit current ( $I_{sc}$ ) as expected. For each filter type the seeding density was  $\sim 350,000$  cells/cm<sup>2</sup>. All cells were initially grown in a medium consisting of a 1:1 mixture of DME and Ham's F-12 supplemented with 50  $\mu$ g/ml gentamicin, 20  $\mu$ g/ml norfloxacin, 5 pM triiodothyronine, 50 nM hydrocortisone, 5  $\mu$ g/ml transferrin, 5  $\mu$ g/ml bovine insulin, 10 nM sodium selenite, and 1% wt/vol bovine albumin (Intergen, Purchase, NY). The cells reached confluence by day 3 at which time hydrocortisone, norfloxacin, and albumin were removed.

For monolayers to be studied by patch clamp, 100 nM aldosterone and 100 nM dexamethasone were added on day 3 and were present continuously to ensure optimal Na<sup>+</sup> transport. In addition to the steroids, 1 mM 4-methylumbelliferyl-D-xyloside and 2.5 mM D(+)-galactosamine were added. We have found that these compounds greatly enhance the rate of seal formation (36), probably due to their ability to inhibit proteoglycan synthesis (37, 38). Pilot studies have shown that they do not alter the qualitative response of the IMCD monolayers to steroids and that they retain their sensitivity to benzamil (data not shown).

For monolayers where the effect of steroid treatment was to be examined, steroid exposure was initiated on day 4 or 5 so that cells had at least 24 h. of steroid-free incubation. Mineralocorticoid (MC) treatment consisted of 100 nM aldosterone and 10  $\mu$ M of the glucocorticoid receptor antagonist RU 38486. Glucocorticoid (GC) treatment consisted of 100 nM dexamethasone and 10  $\mu$ M of the mineralocorticoid receptor antagonist spironolactone (1, 39). Control monolayers received only the carrier (ethanol).

The transmonolayer  $I_{sc}$  and transepithelial resistance measurements

were made in a modified Ussing chamber as previously described (1, 2, 36) on the day the cells were to be used. For each isolation used for mRNA analysis, parallel cultures were made in the large and the small Millicell filters. The large filters were used for RNA isolation and the small filters used for the electrical measurements to be sure that the values were typical of those we have previously reported (1, 2, 35, 36). The mean  $I_{sc}$  value for the Transwell-clear® filters, measured on each filter before use in the patch clamp experiments was 8.0  $\mu$ A/cm<sup>2</sup> and was in the range expected for cells cultured on Millicell filters.

**Patch clamp analysis.** Pipettes were constructed from thin-wall single-barrel borosilicate glass tubing (World Precision Instruments, Sarasota, FL) using a P-87 Flaming-Brown micropipette puller (Sutter Instrument, San Rafael, CA). They were coated with a layer of Sylgard 184 (Dow Corning, Midland, MI) and fire polished to a resistance between 6 and 10 M $\Omega$ . Filters were cut from the cups and held down by a small plastic "donut" on a glass-bottom microchamber so that the apical membranes of the monolayer faced upward. The cells were constantly superfused with a bathing solution maintained at 35°C on a thermal stage (Brook Industries, Lake Villa, IL). The patch clamp pipette was directed by a PCS-100 piezo drive patch-clamp micromanipulator (Burleigh Instruments, Fishers, NY) to make contact with the apical membrane. Seals having a resistance greater than 10 G $\Omega$  were considered acceptable.

Three configurations were used to record single channel currents (40). Cell-attached patches were used to detect channel activity in intact cells. These patches were sometimes excised to create inside-out patches where the cytoplasmic surface was exposed to the bath solution. We also used the outside-out configuration to examine the effect of Na<sup>+</sup> channel blockers. This configuration was achieved by first obtaining an acceptable seal, applying suction to rupture the patch, and pulling the pipette slowly away from the cell so that a portion of the cell membrane would reseal such that the extracellular surface faced the bath solution.

Command voltages were generated and membrane currents were acquired with an Axopatch 200 amplifier and CV201 headstage through a Labmaster TM 125 analog-to-digital converter under the control of pClamp software v6.02 (Axon Instruments, Burlingame, CA). Currents were routinely acquired at 1 kHz and filtered at 200 Hz directly to the computer and hard disk. All data was simultaneously recorded on a VCR-based PCM recorder (A.R. Vetter Co., Rebersberg, PA). For the detailed kinetic analysis of the 3 single channel patches, the currents were reacquired from tape at 10 kHz and filtered at 1 kHz. The pClamp software was used for calculating slope conductance, open state probability ( $P_o$ ), and mean open and closed times. In patches with multiple channels,  $P_o$  was estimated from the expression  $NP_o = \sum_{n=0}^N \frac{nt}{T}$ , where  $n$  is the number of channels open,  $t$  is the time during which  $n$  channels were open,  $T$  is the total recording time and  $N$  is the total number of channels in the patch. This latter value was estimated from maximum and minimum current values and may underestimate the actual number of channels.

We used standard voltage and current conventions. In cell-attached patch configuration, the reported voltage is the bath voltage with respect to the pipette. Thus a negative voltage in this configuration represents membrane hyperpolarization. In the excised-patch configuration, the reported voltage is referenced to the pipette; thus a reported voltage of  $-60$  mV represents a physiologically normal state. In the outside-out patch configuration, voltage is referenced to the bath. Thus in each configuration the voltage orientation reflects the physiologic state. Positive ions entering ions entering the cell are defined as inward currents and are always shown as downward deflections.

**Solutions for patch clamp experiments.** The standard bathing solution contained (in mM) 140 NaCl, 4.5 KCl, 2.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Hepes, 5 D-glucose, pH 7.35. The pipette solution contained (in mM) 140 LiCl, 3 MgCl<sub>2</sub>, 10 Hepes, pH 7.35. Li<sup>+</sup> was used in place of Na<sup>+</sup> because Na<sup>+</sup> selective channels have a higher single channel conductance to Li<sup>+</sup> facilitating single channel analysis (20, 27). The bath K<sup>+</sup> aspartate solution contained (in mM) 140 K<sup>+</sup> aspartate, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Hepes, pH 7.2. The pipette solution for the outside-out

configuration contained 140 KCl, 1 MgCl<sub>2</sub>, 0.55 CaCl<sub>2</sub>, 1 EGTA, 4 Na<sub>2</sub>ATP, 10 HEPES, pH 7.2. The calculated free [Ca<sup>2+</sup>] for this solution was 100 nM (41). The bath solution for the outside-out configuration was the LiCl pipette solution used for the cell-attached and excised patch experiments.

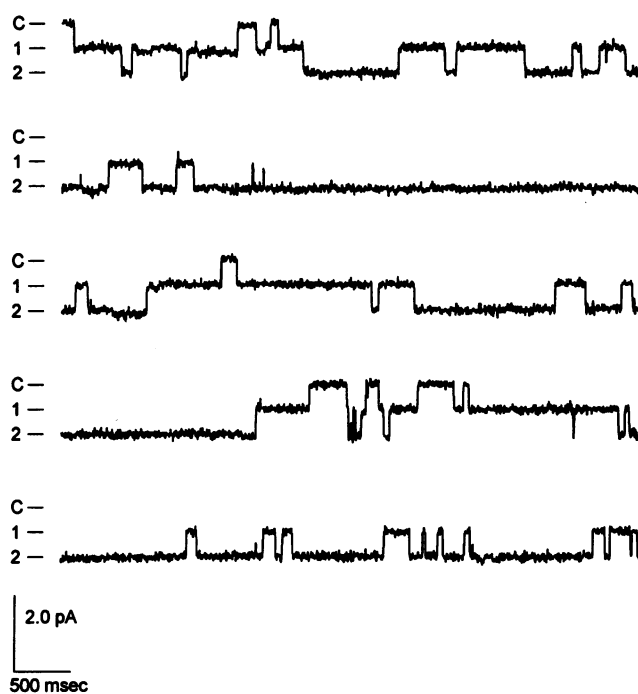
**Preparation of RNA and Northern analysis.** Pieces of kidney cortex, outer medulla, and the entire inner medulla (papilla) were rapidly dissected and frozen in liquid nitrogen. Total RNA was isolated from IMCD monolayers grown on 30-mm filter cups or from regions of the kidney using the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (42). Each 30 mm filter cup yielded ~20–25 µg RNA and a single papilla yielded a similar amount. This amount of extracted RNA was dissolved to a concentration of 2 µg/µl in water and denatured with 20 µl of buffer containing 72% deionized formamide, 10% formaldehyde, and 15 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5) at 65°C for 5 min. The gel-loading buffer consisted of 50% glycerol, 1 mM EDTA, 100 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5) and 0.25% bromophenol blue was added (to 0.1 volume), and ethidium bromide was added to a final concentration of 0.03 mg/ml. The samples were fractionated by electrophoresis through a 1.5% agarose/6.6% formaldehyde gel buffered with 10 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.5) using a Hoefer HE-99 horizontal submarine unit (Hoefer Instruments, San Francisco, CA) at 100 V for 90 min. at 18–20°C. The gel was photographed under ultraviolet light for subsequent analysis by densitometry. The RNA was then transferred onto nylon (Hybond N; Amersham, Arlington Heights, IL) by capillary transfer in 10× standard saline citrate (SSC) and UV crosslinked (UV Stratalinker; Stratagene, La Jolla, CA). Blots were prehybridized and hybridized in a buffer containing 1% bovine serum albumin (Boehringer-Mannheim), 1 mM EDTA, 500 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.2), and 7% sodium dodecyl sulfate (SDS) according to Church and Gilbert (43).

The fragments of cDNA used to probe the blot were amplified by polymerase chain reaction from rat kidney cDNA (Clontech, Palo Alto, CA). The α subunit was a 959-bp segment obtained using PCR with primers designed from the published cDNA sequence (19) as previously reported (24). β and γ subunit fragments were generated by PCR using degenerate primers designed from the published amino acid sequences (20) as previously described (25). The γ fragment (1224 bp) was subcloned into pCRII (Invitrogen) and used as a probe for these studies. The β fragment was generated by PCR as previously described (25) and was extended by 3' rapid amplification of cDNA (GIBCO-BRL) using the primer 5'-GACTGAATTTGGGTCTCAAGTTGATCCT-3' to give a 1500 bp cDNA which was subcloned into pCRII.

Each subunit probe was excised from the cloning vector using the appropriate restriction endonuclease and gel purified using GeneClean (Bio 101, Vista, CA). The probes (100 ng) were radiolabeled with [<sup>32</sup>P]dCTP (3000 Ci/mole) using a random prime DNA labeling kit (Boehringer-Mannheim). Each reaction was column purified using Sephadex G-50 (Pharmacia, Piscataway, NJ) and specific activity ascertained (~10<sup>9</sup> cpm/µg). The hybridization solution contained ~10<sup>8</sup> cpm in a total volume of 20 ml. After an overnight hybridization at 65°C, the membranes were washed twice in 2× SSC and 0.1% SDS at 65°C for 15 min. and then twice in 0.5× SSC and 0.1% SDS at 65°C for 30 min.

After hybridization, autoradiography was performed at -85°C for 3–18 d using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) and Cronex Lightning Plus® intensifying screens (DuPont-NEN, Wilmington, DE). The autoradiograms were quantitated with a PDI scanning densitometer using Quantity One software (Huntington Station, NY). The exposure time of the autoradiograms was adjusted so that the density of each of the bands on a given gel fell into the linear range of the instrument.

After quantitating the amount of rENaC subunit mRNA in the cultured IMCD cells, each blot was reprobed with a random-primed 316-bp cDNA probe for rat glyceraldehyde phosphate dehydrogenase (GAPDH) using the same conditions described for the rENaC subunits. The blots were autoradiographed for 1–3 d (without intensifying screens) and quantitated. These values were used to normalize for variations in the amount of RNA loading. There was no effect of steroids on GAPDH



**Figure 1.** Continuous tracing in a cell-attached patch of apical membrane containing at least two active channels. Command voltage is -20 mV (hyperpolarized cell) and channel openings are downward deflections. Pipette solution was LiCl. C indicates the closed state. Currents were acquired at 1 kHz and digitally filtered at 200 Hz.

when normalized for the density of the ethidium bromide stain of the 28 s ribosomal band.

**Materials.** Culture medium and gentamicin were purchased from the University of Iowa Cancer Center. Benzamil was a generous gift from Merck (West Point, PA). Dr. Christie Thomas kindly provided the rat GAPDH cDNA. Unless otherwise indicated, all other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

## Results

**Properties of single ion channels in cell-attached patches.** Fig. 1 shows an example of the type of channel activity we observed in the cell-attached patch configuration. A characteristic feature was the relatively slow kinetics with long-lived openings and closings. Channels with these properties were observed in 7% of acceptable seals (Table I). The current-voltage (I-V) rela-

**Table I.** Frequency of Observing Selective or Nonselective Cation Channels in Apical Membrane as a Function of Patch Configuration

Patch configuration	Selective	Nonselective	
Cell attached	21/298 (7%)	0/298	<i>P</i> < 0.001
Excised patch	0/134	32/134 (24%)	<i>P</i> < 0.001

All excised (inside-out) patches reported here had no detectable channel activity in the cell attached configuration. Cell-attached patches having channel activity were usually not excised (with one exception, Fig. 8). Significance by chi-squared analysis.

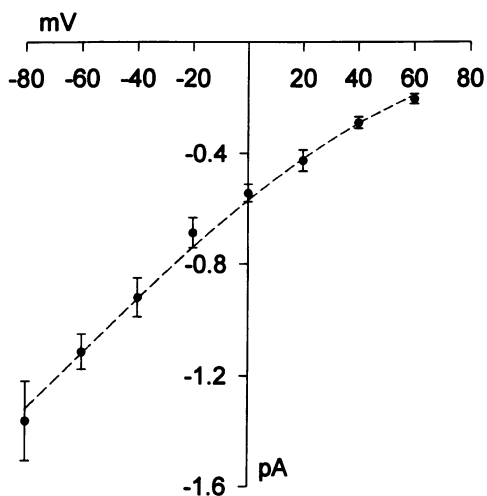


Figure 2. Current-voltage relationship of single channel records compiled from six cell-attached patches. The dotted line represents the expected currents calculated from the best fit to the Goldman flux equation (44). For this fit we assumed a minimal selectivity of  $\text{Li}^+$  over  $\text{Na}^+$  and a membrane voltage of  $-45$  mV. Using these parameters, the calculated intracellular  $[\text{Na}^+]$  was  $8.3$  mM. A nearly identical fit was obtained assuming a membrane voltage of  $-50$  mV. Using this value, the calculated intracellular  $[\text{Na}^+]$  was  $18$  mM. Thus when we assume that  $\text{Na}^+$  and  $\text{Li}^+$  are the dominant charge carriers, we obtain an excellent fit with the predicted behavior and expected intracellular  $[\text{Na}^+]$  (45, 46).

tionship compiled from six patches is shown in Fig. 2 and demonstrates inward rectification (dotted line), consistent with a channel whose permeant ions are present at high concentrations in the pipette but low in the cell (44). The reversal voltage ( $E_{\text{rev}}$ ) is more positive than  $+80$  mV. Assuming a membrane voltage of  $-50$  mV (10), and intracellular ion concentrations that have been reported for rat IMCD cells (i.e.,  $[\text{Cl}^-] \sim 70$  mM,  $[\text{K}^+] \sim 130$  mM, and  $[\text{Na}^+] \sim 25$  mM, (45–46), we conclude that the channels are highly selective for  $\text{Li}^+$  over  $\text{K}^+$  and over  $\text{Cl}^-$ . The channel's slope conductance calculated from this  $I$ - $V$  plot was  $11.1$  pS in the hyperpolarized range ( $-80$  to  $-20$  mV) and  $6.6$  pS in the range of the resting membrane voltage ( $-20$  to  $+20$  mV).

In seven patches containing three or fewer channels, the open state probability ( $P_o$ ) at  $0$  mV ranged from  $0.32$  to  $0.86$  with a mean ( $\pm$ SEM) value of  $0.60 \pm 0.08$ . There was no evidence for a voltage-dependence to  $P_o$  at command voltages between  $-80$  and  $+40$  mV (Fig. 3), an observation similar to the  $\text{Na}^+$  channel in rat CCD (27), rabbit CCD (26), and rENaC expressed in oocytes (20).

Another set of important kinetic characteristics, the mean open and closed times, can be most accurately determined in patches that contain only one channel. Because many of the patches contained several channels, we made an initial estimate of the mean open time using stretches of recordings where only one channel appeared to be active. The mean open times for 15 such channels ranged from  $20$  to  $2733$  ms.; the median value was  $319$  ms. and the mean value was  $666$  ms. We analyzed in greater detail the mean open and closed times in three patches that contained only one detectable channel over the entire recording period and where the duration of the seal was sufficiently long to obtain accurate estimates. The plot of the compiled log mean open and closed times for a representative chan-

nel is shown in Fig. 4. The average mean open time values for these three were  $280$ ,  $406$ , and  $666$  ms. The mean closed time analysis showed two states with longer values of  $525$ ,  $627$ , and  $892$  ms. and a shorter value of  $< 10$  ms (Fig. 4 B).

In order to examine the sensitivity to amiloride and benzamil, we used the outside-out configuration. The advantage of this approach is that one can change the bath solution more easily than the pipette solution and thus improve the probability of demonstrating recovery. Another advantage is that outside-out patches usually have a larger membrane area than cell-attached patches and therefore there is a higher probability of detecting channel activity. Fig. 5 shows the results of an experiment in which amiloride ( $1 \mu\text{M}$ ) reversibly reduced the mean current and produced the typical flickering block demonstrated by other investigators for  $\text{Na}^+$  selective channels (26, 28, 47). After removing amiloride and allowing channel activity to recover, we added benzamil, a more specific inhibitor of  $\text{Na}^+$  selective channels (5). Benzamil inhibited virtually all channel activity.

**Nonselective cation channel.** The characteristics of the channel shown in Figs. 1–5 are quite different from those of the non-selective cation channel previously proposed to be responsible for  $\text{Na}^+$  entry across the apical membrane of the IMCD (6, 7, 48). We never observed non-selective channels in cell-attached patches (Table I). However, to determine if these IMCD cells contained non-selective cation channels, we studied excised apical membrane patches because the properties of nonselective cation channels in IMCD cells have usually been studied in this configuration (6, 7, 9, 48). We excised patches only if they had no detectable channel activity while cell-attached (with one exception, *vide infra*). Upon excision (into a high Ca bath to ensure detection), non-selective channels appeared in 24% of the patches (Table I). A characteristic tracing is shown in Fig. 6.

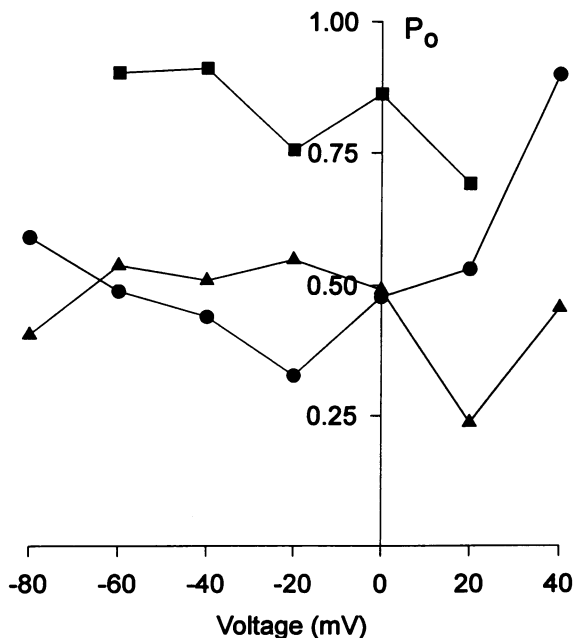
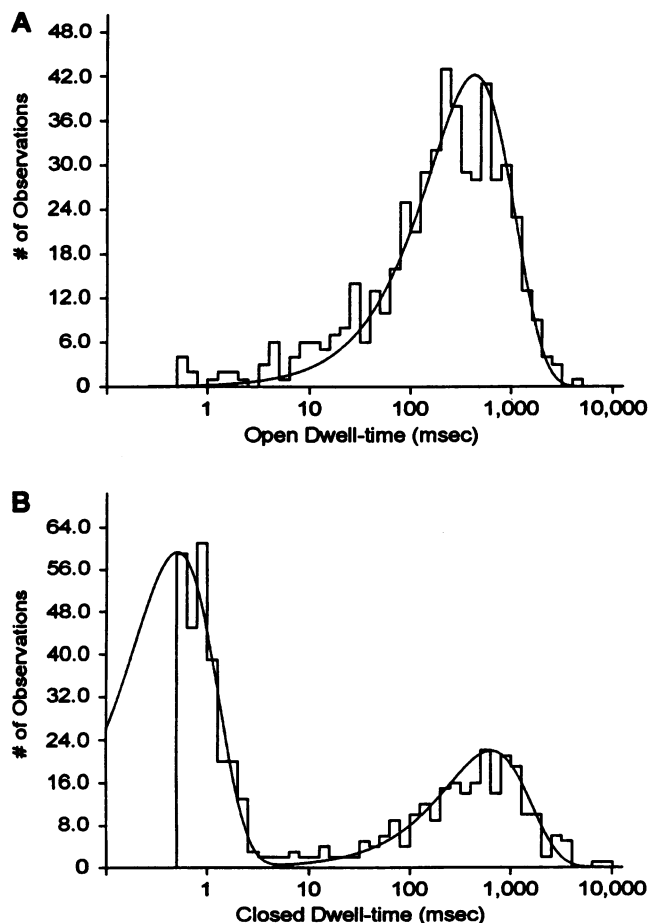


Figure 3. Open state probability ( $P_o$ ) of cell-attached single channels as a function of command voltage. Each line represents one patch containing several channels.



**Figure 4.** Kinetics of a representative channel. (A) Open dwell time histogram of a cell-attached single channel. Time is displayed as a logarithmic function. Mean open time for this channel was 406 ms. (B) Closed time histogram for the same channel. Mean closed time for the longer value was 627 ms. Currents were acquired at 10 kHz and filtered at 1 kHz. Transitions shorter than 0.5 ms. were ignored.

The  $I-V$  relationship for this type of channel was linear (Fig. 7). When excised into the bath solution (140 mM NaCl), the single channel conductance was 30.1 pS. When the bath was changed to one containing 140 mM  $K^+$  aspartate, the single channel conductance was 26.5 pS and the  $E_{rev}$  (after correction for the liquid junction voltage) did not change. Thus the permeabilities of  $Li^+$ ,  $K^+$ , and  $Na^+$  were similar.

The difference between the non-selective cation channel and the low conductance  $Na^+$  selective channel was highlighted in a single experiment (Fig. 8). We observed an active,  $Na^+$  selective channel in the cell-attached configuration. After the patch was excised into the standard bath solution, the selective channel maintained its activity, and another channel became active. The newly activated channel had the characteristics of the non-selective cation channel. This experiment shows that these two types of channel can coexist under identical conditions in the same patch.

**rENaC mRNA in inner medulla and regulation by steroid hormones.** The characteristics of the  $Na^+$  selective channel in the apical membrane are similar to those reported for rENaC expressed in oocytes (19). In order to determine if rENaC might be responsible for the channel activity, we first asked whether

rENaC subunit mRNAs were present in the renal inner medulla. Northern blot analysis on RNA isolated from renal cortex, outer medulla, and inner medulla showed that all three subunit mRNAs were detected in all regions (Fig. 9). Each probe identified a message of different size corresponding to that reported by Canessa (20). The cortex appeared to have the highest relative abundance of all three subunits when normalized for the amount of RNA loaded as estimated by the intensity of the ethidium bromide stain of the 28 s ribosomal band.

Because steroid hormones increase  $Na^+$  transport by the IMCD (1, 2), we asked whether they increase the amount of rENaC subunit mRNA. Fig. 10 shows a representative set of Northern blots using RNA isolated from primary cultures demonstrating effects of either GC or MC on the abundance of each of the subunit mRNAs after 3, 8, or 24 h. of steroid exposure. Fig. 11 shows the densitometric quantitation of all the blots. There was no effect of either GC or MC on the  $\beta$  or  $\gamma$  subunit mRNA. In contrast, GC increased the  $\alpha$  subunit mRNA abundance an average of 6.9-fold and MC increased the abundance an average of 4.6-fold.

## Discussion

We conclude that rENaC is the predominant channel responsible for  $Na^+$  absorption in the rat IMCD based on three observations. First, the type of channel we detect in the apical membrane in the cell-attached configuration (Fig. 1) has characteristics similar to those detected when rENaC is expressed in oocytes (20). Second, mRNA for each of the three rENaC subunits is present in the renal papilla (Fig. 9) and in primary cultures of IMCD cells (Fig. 10). Third, steroid hormone exposure increases  $Na^+$  transport by the IMCD (1, 2) and the abundance of  $\alpha$ -rENaC mRNA (Fig. 11).

**Characteristics of the apical membrane  $Na^+$  channel.** Our results are the first to demonstrate highly selective  $Na^+$  channels in the apical membrane of the IMCD cells. At least 3 other groups of investigators have studied single channels in IMCD cell membranes; all have reported non-selective cation channels (7, 9, 18). We attribute the difference in these results to at least three factors. The first factor is the patch configuration. With few exceptions, (Figs. 6 and 8) we studied  $Na^+$  selective single channels while cell-attached. It is possible that highly selective  $Na^+$  channels are less active and more difficult to detect after excision while nonselective cation channels are more readily detected. The second factor is the temperature. By using nearly physiologic temperature (35°C), instead of room temperature, we may have encouraged a relatively greater channel activity. The third factor is the nature of the preparation. Whereas others have studied IMCD cells grown on plastic or glass, we grew the cells on filters. Hamilton and Eaton (49) have reported that A6 cells grown on filters have more highly selective  $Na^+$  channels than cells grown on plastic. It is possible that epithelial cells grown on solid supports have a less-well differentiated apical and basolateral membrane, thus adding to the difficulty in detecting highly selective  $Na^+$  channels. An additional advantage to growing the cells on filters is that we document the presence of a representative  $I_{SC}$  in each monolayer studied. With this measurement we can be sure that  $Na^+$  channels are present in the apical membrane of at least some cells.

The single channel properties of the selective  $Na^+$  channel of the IMCD are similar to those of rENaC expressed in oocytes (20, 50). Both have relatively long mean open and closed times

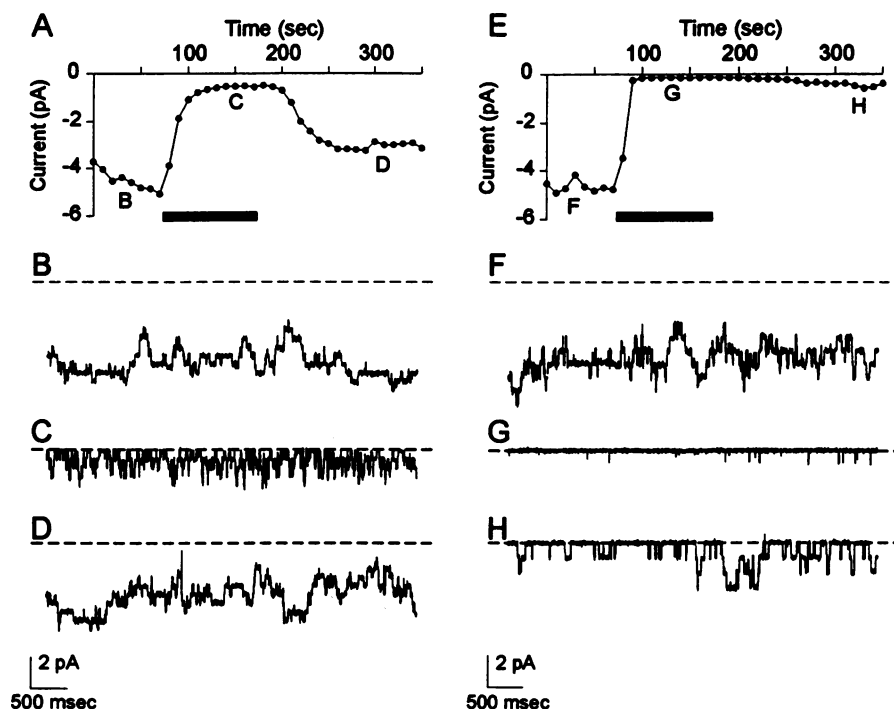


Figure 5. Effect of  $\text{Na}^+$  channel blockers on activity of an IMCD apical membrane patch in the outside-out configuration. At least 10 channels were present in this patch. The I-V plot demonstrated a slope conductance and  $E_{rev}$  similar to Fig. 2 (not shown). Panel A shows 10-s mean current values at a holding voltage of  $-30$  mV before amiloride (B), during  $1 \mu\text{M}$  amiloride in the bath (bar C), and after washout (D). Panels B, C, and D show examples of channel activity during each of the three periods. Dashed lines indicate the current level when all channels were closed. Panel E shows a similar plot  $\sim 3$  min. after amiloride washout before (F), during (bar G), and after (H)  $10 \mu\text{M}$  benzamil addition (in the same patch). Holding voltage for this series was  $-60$  mV. Panels F, G, and H show examples of channel activity during each of these periods. Note the slow recovery from benzamil.

(Fig. 4), similar single channel conductances with  $\text{Li}^+$  in the pipette (Fig. 2), high selectivity for  $\text{Na}^+$  (Fig. 2), and  $P_o$  values that are relatively insensitive to membrane voltage (Fig. 3). These similarities provide good, but not irrefutable, evidence that rENaC is the predominant  $\text{Na}^+$  channel in the IMCD cell. Differences in experimental conditions, differences in the expressing cell (oocyte vs. rat IMCD), and sampling errors owing to limited numbers of channels reported in oocytes (20) might produce fortuitous similarities. However, there is good evidence that these similarities are not just coincidental. At least four other tissues have highly selective apical membrane  $\text{Na}^+$  channels with these properties; CCD principal cells from salt-deprived rats (27, 28), aldosterone treated primary cultures of rabbit CCD (26), *Ambystoma* collecting duct (47), and aldosterone treated A6 cells (29).

Comparison of the characteristics of rat CCD and IMCD  $\text{Na}^+$  selective channels is relevant and instructive. Both are permeant to  $\text{Li}^+$  (27), both are amiloride sensitive (28, Fig 5), both tend to occur in multichannel clusters (28, Fig. 1), both have  $P_o$  values that are variable but relatively insensitive to

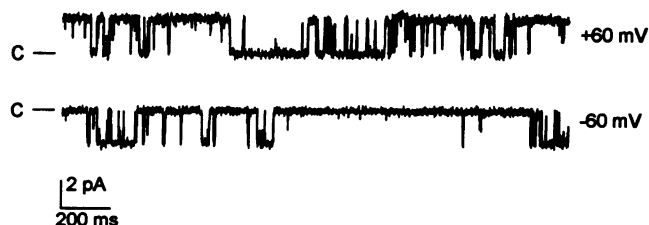


Figure 6. Representative tracing of a nonselective cation channel in an apical membrane patch excised into the  $140$  mM  $\text{Na}^+$  bath solution. Pipette solution was  $\text{LiCl}$ . Note time scale is different than that of Fig. 2. C indicates closed state; holding voltage is indicated to the right of each sweep.

voltage (27, Fig. 3), and both have single channel conductances of  $11.1$  pS with  $\text{Li}^+$  in the pipette at hyperpolarizing voltages (27, Fig. 2). The kinetics of the two channels are also similar, although the CCD data were conducted at room temperature. Both channels have variable mean open times that suggest a single open state typically greater than  $300$  ms, and both have

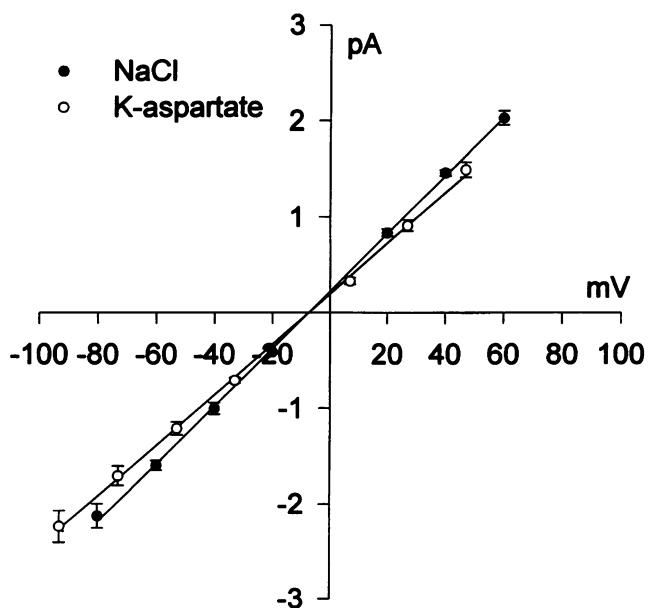


Figure 7. I-V plot of the non-selective cation channel. Channel activity was only seen in the excised patch configuration. Closed circles represent data from the  $140$  mM  $\text{NaCl}$  bath; open circles from the  $140$  mM  $\text{K}^+$  aspartate bath. Data for the  $\text{K}^+$  aspartate bath are corrected for the liquid junction voltage (measured value of  $13$  mV). Values are mean  $\pm$  SEM of five excised patches.

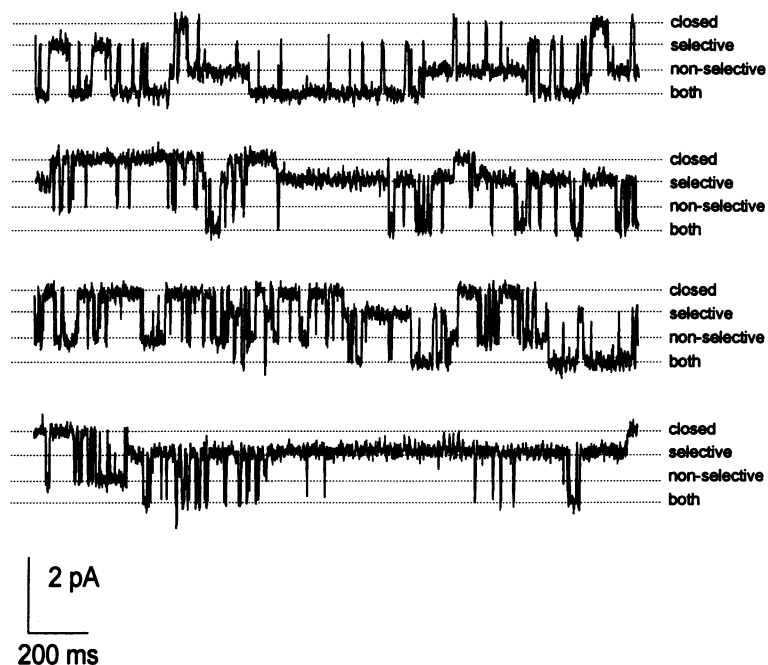


Figure 8. Simultaneous activity of the non-selective cation channel and the Na<sup>+</sup> selective channel in an excised patch. Channel openings are down. In these representative tracings where the holding voltage was -60 mV, one can detect activity of the Na<sup>+</sup> selective channel which displays the slow kinetics also demonstrated in the cell-attached configuration. In addition, the nonselective cation channel, which was not detected in the cell-attached configuration, is easily recognized by its typical kinetics and larger conductance. "Both" indicates the current level at which both channels are open.

mean closed times that suggest two states with the slower component longer than 500 ms (28, Fig. 4). The tendency to find highly selective Na<sup>+</sup> channels in clusters is also characteristic of A6 cells and rabbit CCD cells in culture (26, 29). Furthermore the kinetic analysis of these Na<sup>+</sup> selective channels after aldosterone exposure shows the characteristic long open times and two closed states. We interpret these data to suggest that the highly selective Na<sup>+</sup> channel in each of these preparations represents the same molecular complex. The presence of mRNA for each of the three rENaC subunits in the rat CCD (32) and the IMCD (Fig. 10) further support the conclusion that this complex is rENaC.

How are we to interpret the role of the nonselective cation channel in Na<sup>+</sup> transport by the IMCD? Based on the absence of the nonselective cation channel in cell-attached patches (Table I), it seems unlikely that it plays a role in steroid-stimulated Na<sup>+</sup> transport in IMCD cells. Other investigators have also come to this conclusion based on its nonselectivity, its nearly ubiquitous distribution, and the fact that its regulation by Ca<sup>2+</sup> and ATP are such that it seems unlikely to be active under normal physiologic conditions (9, 18). The demonstration that

both channel types can exist in the same excised patch (Fig. 8) suggests that the nonselective cation channel is not a rENaC molecule that invariably operates in a nonselective mode upon excision. The exact role of the non-selective cation channel remains unclear.

*rENaC and Na<sup>+</sup> transport along the collecting duct.* Recently, Duc et al. (32) reported that all three subunits of rENaC were detected by in situ hybridization and immunocytochemistry in the rat CCD and outer medullary collecting duct, but not in the IMCD. They proposed two possible explanations for this heterogeneity; the Na<sup>+</sup> channel in the IMCD might be another molecule, or rENaC might not be detected because of low abundance or another technical reason. Our results suggest that a major reason for their results is the low abundance. The Northern blots of the three regions of the kidney (Fig. 9) show that the cortex had the greatest amount of mRNA for all three rENaC subunits when normalized for differences in loading using the density of the 28 s ribosomal band. Since the relative mass of collecting duct cells is lowest in the cortex and highest in the medulla (51), we conclude that the IMCD contains less rENaC mRNA per unit tubule length than does the collecting duct from the cortex or the outer medulla.

This difference in subunit mRNA abundance between the CCD and the IMCD compares to axial differences in Na<sup>+</sup> transport capacity along the collecting duct as assessed using freshly dissected, perfused tubules. Whereas the CCD dissected from rats (or rabbits) pretreated with MC agonists transport large amounts of Na<sup>+</sup> (52–55), the IMCD dissected from similarly treated rats (10, 56) or hamsters (57) transports small amounts of Na<sup>+</sup>. This decline in maximum Na<sup>+</sup> transport capacity (at least in the rabbit) begins in the outer medullary collecting duct (52). Thus the intrinsic capacity to transport Na<sup>+</sup> as a function of axial position appears to correlate with the abundance of the subunit mRNA along the length of the collecting duct.

*Implications of rENaC mRNA regulation for epithelial Na<sup>+</sup> transport.* Stimulation of the α-rENaC subunit mRNA by steroids (Fig. 11) has also been reported in the lung (21, 34).

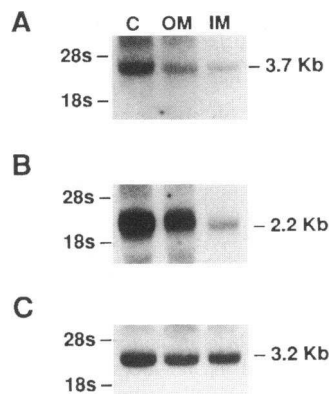
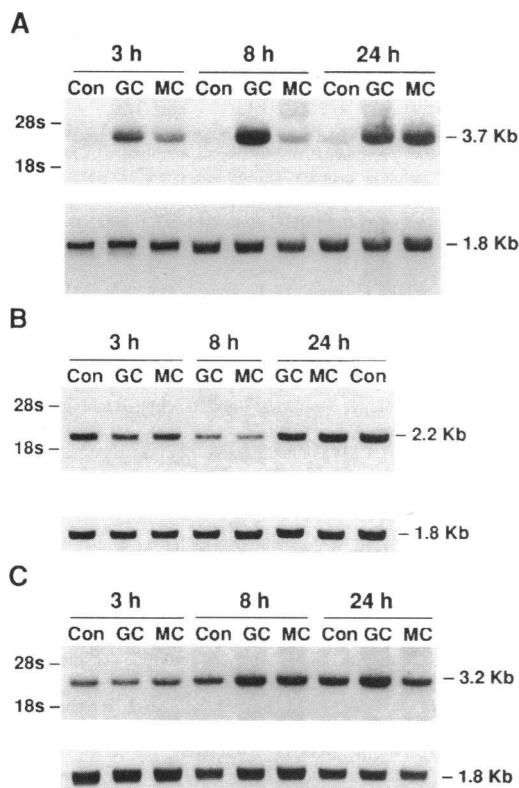


Figure 9. Northern blot of rENaC subunits from renal cortex (C), outer medulla (OM), and inner medulla (IM) from normal Wistar rats. Separate blots were probed with the random-primed cDNA for alpha (A), beta (B), and gamma (C) subunits. Exposure was 18 d for each blot.



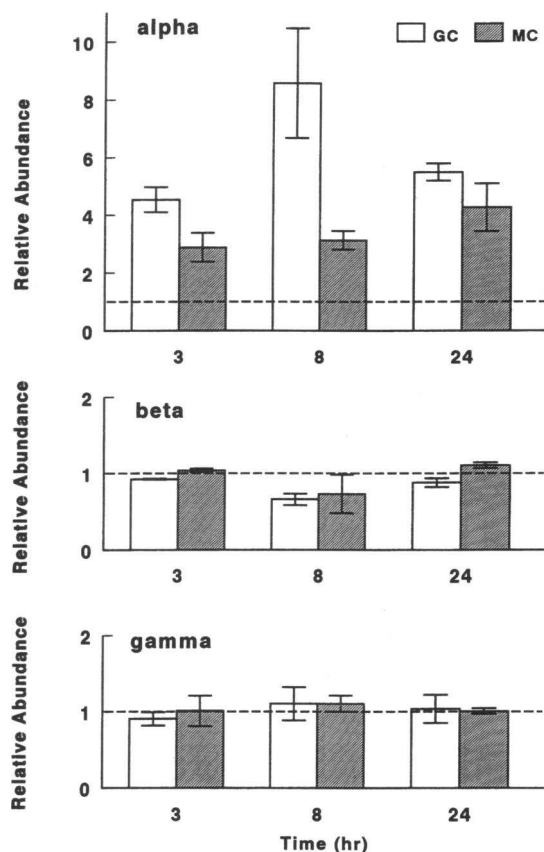


**Figure 10.** Representative Northern blot demonstrating rENaC subunit mRNA from IMCD primary cultures exposed to glucocorticoid (GC) or mineralocorticoid (MC) hormone. The upper panels represent alpha (A), beta (B), or gamma (C) subunit. The lower panels show the 1.8-kb GAPDH probe used to normalize for loading.

Using cultured distal fetal rat lung epithelial cells, Champigny et al. (33) demonstrated an increase in  $\alpha$ -rENaC mRNA in response to dexamethasone and to aldosterone. However, the effect of aldosterone in the lung was mediated via the glucocorticoid receptor rather than the mineralocorticoid receptor because the effect was blocked by the glucocorticoid receptor antagonist RU 38486. This result is clearly different from the IMCD where RU 38486 did not block the aldosterone effect (Fig. 11). The simplest explanation for these differences is that the IMCD contains mineralocorticoid receptors and the distal fetal lung does not.

The observation that GC and MC increase the abundance of  $\alpha$ -rENaC mRNA but not  $\beta$ -rENaC or  $\gamma$ -rENaC mRNA (Fig. 11), suggests that the transcriptional regulation of the  $\alpha$ -rENaC subunit, or perhaps a post transcriptional modification of  $\alpha$ -rENaC mRNA, might be a rate-limiting step in the production or assembly of a fully functional  $\text{Na}^+$  channel. This analysis has not yet been completed on other subunits in the lung, but the effect of GC on  $\alpha$ -rENaC mRNA levels is consistent with this possibility.

Despite the fact that both the lung and the IMCD increase the levels of  $\alpha$ -rENaC mRNA in response to steroids, not all cells expressing steroid-responsive epithelial  $\text{Na}^+$  channels behave identically; the colon, for example, appears to be different. In an effort to elevate circulating levels of aldosterone by the physiologic maneuver of NaCl restriction, Lingueglia et al. (22) have reported no effect on the abundance of  $\alpha$ -rENaC mRNA in colon. Rather, there was an increase in the abundance of  $\gamma$ -



**Figure 11.** Quantitative expression of the effect of glucocorticoid (GC) or mineralocorticoid (MC) on the relative mRNA abundance of alpha, beta, or gamma subunit. Open bars indicate GC exposure, hatched bars indicate MC exposure. Dashed line represents untreated control normalized to the GAPDH signal and defined as unity.  $n = 3, 2,$  and  $3$  sets respectively for alpha, beta, and gamma subunits. There was no significant effect of GC or MC on relative beta or gamma subunit abundance. There was a strong effect of both GC and MC on the alpha subunit ( $P < 0.0001$ ). The effect of GC was greater than MC ( $P < 0.001$ ) and there was no significant effect of time on the action of either GC or MC (by ANOVA).

rENaC mRNA. The simplest explanation for these observations is that the steroid effect on rENaC subunit mRNA is tissue specific. An alternative possibility is that NaCl restriction produces a more complex sequence of physiologic events than just raising circulating aldosterone concentrations. Of course, these possibilities are not mutually exclusive.

What are the implications of having rENaC in both the CCD and IMCD for normal  $\text{Na}^+$  and  $\text{K}^+$  homeostasis? In the CCD,  $\text{Na}^+$  absorption is linked to  $\text{K}^+$  secretion and both processes are increased by aldosterone. This linkage occurs because the CCD principal cell apical membrane has both rENaC and  $\text{K}^+$  channels which direct  $\text{K}^+$  into the lumen (58). In contrast to the CCD, the IMCD does not have significant apical  $\text{K}^+$  channel activity and thus normally secretes little  $\text{K}^+$ . It is conceivable that different local factors determine the responsiveness of the CCD and the IMCD to aldosterone (59). A relatively more responsive CCD would produce an antinatriuresis and a kaliuresis while a relatively more responsive IMCD would produce an antinatriuresis without a kaliuresis.

The recent discovery that Liddle's syndrome is caused by

a truncation of the terminal portion of the  $\beta$ -rENaC subunit (60) firmly identifies rENaC as a critical component of  $\text{Na}^+$  homeostasis and blood pressure control. The syndrome was discovered because affected family members were hypertensive, hypokalemic, had low plasma renin activity and low aldosterone levels, were unresponsive to mineralocorticoid receptor antagonists but responsive to  $\text{Na}^+$  channel antagonists (61). This constellation of findings suggested that there was overactivity of the CCD  $\text{Na}^+$  channel. While most hypertensive patients are not hypokalemic, a large fraction have low renin and aldosterone. We postulate that some patients with low renin hypertension might have a rENaC that is dysregulated in the IMCD rather than in the CCD. In this regard, it will be important to further understand the details of rENaC regulation in various target organs as well as within the kidney.

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