J. Membrane Biol. 156, 9–18 (1997)

The Journal of
Membrane
Biology

The Basis of Higher Na^+ Transport by Inner Medullary Collecting Duct Cells from Dahl Salt-Sensitive Rats: Implicating the Apical Membrane Na^+ Channel

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Received: 6 May 1996/Revised: 16 October 1996

Abstract. The present experiments were designed to examine the function of Na/K pumps from Dahl salt-sensitive (S) and salt-resistant (R) rats. Previous reports have suggested that there is a difference in primary sequence in the α_1 subunit, the major Na/K pump isoform in the kidney. This sequence difference might contribute to differences in NaCl excretion in these two strains which in turn could influence the systemic blood pressure. Using ''back-door'' phosphorylation of pumps isolated from basolateral membranes of kidney cortex, we found no differences between S and R strains. We also examined the Na/K pumps from cultured inner medullary collecting duct (IMCD) cells. This approach takes advantage of the fact that monolayers cultured from S rats transport about twice as much Na^+ as monolayers cultured from R rats. In cells whose apical membrane was made permeable with amphotericin B, comparison of the affinities for ouabain, Na^+, and K^+, respectively, showed only small or no differences between S and R monolayers. Ouabain binding showed no difference in the number of Na/K pumps on the basolateral membrane of cultured cells, despite a 2-fold difference in Na^+ transport rates. The analysis of the steady-state Na^+ transport indicates that Na/K pumps in IMCD monolayers from S rats operate at a higher fraction of their maximum capacity than do pumps in monolayers from R rats. The results, taken together, suggest that the major reason for the higher rate of Na^+ transport in S monolayers is because of a primary increase in the conductive permeability of the apical membrane to Na^+. They suggest that the epithelial Na^+ channel is intrinsically different or differently regulated in S and R rats.

Key words: Na^+ transport — Salt-sensitive rats — Apical membrane — Na/K pumps — IMED cells — Kidney cortex

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Introduction

The inbred strains of Dahl rats whose blood pressure is sensitive (S) or resistant (R) to a high NaCl diet have provided a superb model for studying some of the physiologic mechanisms responsible for hypertension. Particularly valuable has been the opportunity to study mechanisms involved in NaCl excretion by these two strains. Many studies have demonstrated that the kidneys of these rat strains participate in the sensitivity or resistance to NaCl loading [36]. The specific abnormalities are incompletely understood, but it seems likely that more than one nephron segment is involved in the inappropriate NaCl retention in the Dahl S rat. There is good evidence that the proximal tubules from S and R rats respond differently to salt loading [26, 30, 32, 41]. In addition, Henle's loops from S rats absorb more NaCl than those of R rats, a property that is independent of the dietary NaCl intake and is detectable before the onset of hypertension [21, 56].

Recently, attention has been directed toward identifying differences in Na^+ transport by the collecting duct of S and R rats. There is evidence that the cortical portion of the collecting duct from S rats absorbs more Na^+ than R rats, but the physiologic significance is unclear [23]. We have recently reported that inner medullary collecting duct (IMCD) cells cultured from prehypertensive Dahl S rats transport twice as much Na^+ as IMCD cells from R rats [19]. The cellular mechanisms are not known, but we have excluded differences in the underlying nature of Na^+ transport (i.e., electroneutral *vs.* electrogenic), availability of metabolic energy, and the capacity for steroid metabolism [19].

One of the differences between Dahl S and R rats that could account for different renal responses to Na^+ loading involves the Na/K pump. Herrera and Ruiz-Opazo [15] have reported a primary sequence difference between the α_1 subunits of S and R rats. Their data suggest a functional difference that could be translated into inappropriate NaCl retention in S rats [4, 15, 34]. It should be noted that the sequence difference is disputed [42, 43, 47, 48], and thus any role assigned to the Na/K pump on this basis must be interpreted with caution. Adding to the complexity of the situation is the fact that the gene for the α_1 pump subunit does not cosegregate with hypertension in S and R backcrosses [37]. Thus, whether the Na/K pump plays a major role in effecting the difference in Na^+ excretion between S and R rats remains unclear.

The purpose of these experiments was to examine the kinetic and functional characteristics of the Na/K pump from Dahl S and R rats to attempt to detect a difference in their behavior. For most of the studies, we examined the pump as it resided in the basolateral membrane of cultured IMCD cells. We reasoned that these cells would provide a unique model to test specific hypotheses regarding which transport process(es) might be implicated in the different Na^+ transport rates and therefore, provide clues as to the pathogenesis of hypertension.

Materials and Methods

The inbred strains of Dahl salt-sensitive (Dahl SS/Jr) and salt-resistant (Dahl SR/Jr) were obtained from two sources. We purchased some rats from Harlan Sprague Dawley (Indianapolis, IN) before February 1993. We also obtained rats from Dr. John Rapp (Medical College of Ohio, Toledo, OH) and started a colony at the University of Iowa. We did not use any rats from Harlan Sprague Dawley during the time the Dahl SS/Jr strain was suspected of being genetically impure [25, 50, 54]. Rats were sacrificed at 4–5 weeks of age having been fed normal rat chow since weaning. The blood pressures of these rats (S and R) are within the normal range [19].

Preparation of Basolateral Membranes

Rats were anesthetized with methoxyflurane and decapitated, the kidneys removed, and rinsed with phosphate buffered saline solution. The renal cortex was dissected, and the membrane fractions were prepared by the method of Scalera et al. [44] with small modifications. All steps were carried out at 4°C. Tissue samples weighing 0.5 to 1.3 g were homogenized in 35 ml sucrose buffer (0.25 M sucrose, 10 mM triethanolamine, 0.1 mM phenyl-methyl-sulfonyl fluoride, pH 7.6) with 20 strokes of a Teflon pestle in a Thomas homogenizer. After 1:2 dilution by sucrose buffer, the homogenate was spun at 2,500 × *g* for 15 min in a Sorvall RC-5C high speed centrifuge using a Sorvall SA-600 rotor. The supernatant was removed and recentrifuged at 20,500 × *g* for 20 min. The fluffy white pellet was resuspended in 25 ml of sucrose buffer and rehomogenized with 20 strokes of the same pestle.

This rehomogenate was fractionated using 8% (v/v) Percoll (Pharmacia, Piscataway, NJ) by centrifugation at 27,500 × *g* for 40 min using a Sorvall SM-24 rotor. Fractions (1 ml) were collected and diluted 1:5 with sucrose-free buffer (10 mM triethanolamine, 0.1 mM phenyl-methyl-sulfonyl fluoride, pH 7.6). Each fraction was spun at 27,500 × *g* for 40 min in a SM-24 rotor, and the fluffy layer on the bottom recovered in 1 ml sucrose-free buffer and stored at −70°C until measurement. Protein was measured by a fluorescent technique as previously described [1, 51] using bovine serum albumin as a standard.

Enzyme Assays

γ-glutamyl transferase activity was assayed spectrophotometrically by measuring the rate of production of p-nitroaniline from γ-glutamyl-p-nitroanilide as previously described [52]. The reaction medium contained 50 mM ammediol (pH 8.6), 4.6 mM γ-glutamyl-p-nitroanilide, 52 mM glycylglycine, 10.4 mM $MgCl_2$, and 1.5-30 μl membrane solution in total volume of 3 ml. The change in absorbance at 405 nm was continuously monitored at 25°C, and relative activity in each fraction was calculated.

Na/K ATPase activity was measured by a modification of the method of Forbush [8]. The reaction solution contained 15 μg membrane protein, 120 mM NaCl, 8.3 mM KCl, 4 mM $MgCl_2$, 60 mM Tris, 1 mM $(Na)_3EDTA$, pH 7.5 in a total volume of 540 μl in the absence or presence of 5 mM ouabain. The mixture was then preincubated with 0.01 mg/ml deoxycholic acid at room temperature for 1 hr. In preliminary experiments, we obtained maximum enzyme activity with this concentration of deoxycholic acid (18% increase *vs.* control, $n = 3$). The reaction was started by the addition of ATP to a final concentration of 4 mM and continued for 10 min. After addition of 1 ml stop solution containing 8% SDS, the released inorganic phosphate was assayed according to Baginski [2] and compared to a standard curve. Na/K ATPase activity was calculated as the difference between ATPase activity with and without ouabain.

Back-door Phosphorylation

The phosphorylation of the Na/K ATPase in the presence of ouabain was performed by a modification of the method described previously [40]. Duplicate 100 μl aliquots of membrane (15 μg protein) in Hepes-Mg^{++} buffer (100 mM Hepes-Tris, 5 mM $MgCl_2$, 0.01 mg/ml deoxycholic acid, pH 7.4) containing various concentrations of phosphate (1–100 μM H_3PO_4) were incubated for 1 hr at room temperature in the presence of 4.8 mM ouabain. For the control reactions, ouabain was omitted and 100 mM NaCl was added. The reaction was initiated by the addition of 20 μCi [^{32}P]orthophosphate which had been pre-filtered through a Millex-GS 0.22 μm filter (Millipore, Bedford, MA). The phosphorylation reaction was quenched after 5 min at room temperature by the successive addition of 50 μg bovine serum albumin, 1.0 ml ice-cold 5% trichloroacetic acid, and 0.1 M H_3PO_4. The tubes were placed on ice for 5 min and then centrifuged for 2 min in an Eppendorf microfuge at 10,000 × *g*. The resulting pellet was washed three times with 1.0 ml 5% trichloroacetic acid, 0.1 M H_3PO_4 at 4°C, and rapidly rinsed with 0.6 ml of 0.15 M KH_2PO_4, pH 2. The final pellet was resuspended in 200 μl of 0.25 M sucrose, 35 mM cetylpyridinium chloride, 100 mM KH_2PO_4/H_3PO_4. An aliquot (160 μl) was counted in a Beckmann liquid scintillation counter. To the remaining 40 μl, β-mercaptoethanol and pyronin Y were added to final concentrations of 128 mM and 10 μg/ml, respectively. Aliquots (25 μl) were analyzed by polyacrylamide gel electrophoresis at acid pH, as described previously [39]. The calculation of pump sites and turnover number were as previously described [40].

Preparation of Monolayers

Primary cultures of IMCD cells were prepared using the hypotonic lysis isolation method as previously described for this laboratory [17,

18, 24]. The inner medulla was dissected, minced, and incubated in an isotonic solution containing 0.1% collagenase for 2–3 hr. The solution was made hypotonic by addition of 2 volumes of distilled water containing 10 μg/ml DNAse and cells were recovered after 2 centrifugation steps. This isolation procedure usually yielded 20–40 monolayers from 6 kidneys per strain.

Cells were seeded onto collagen-coated polycarbonate filters glued to plastic cylinders as described [17, 18] or they were seeded onto similarly treated 12 mm Millicell PCF filters (Millipore). Seeding density was 20 μg DNA/filter (~350,000 cells/cm^2). Cells were grown for 3 days in a medium composed of a 1:1 mixture of DMEM and Ham's F-12 supplemented with 50 μg/ml gentamicin, 5 pM triiodothyronine, 50 nM cortisol, 5 μg/ml transferrin, 5 μg/ml bovine insulin, 10 nM Na^+ selenite, and 1% wt/vol bovine albumin. On the third day the medium was changed to one from which cortisol and albumin were omitted. After 24 hr in the steroid-free medium the monolayers were confluent as evidenced by the transmonolayer electrical resistance (R_T) being >100 $\Omega \cdot cm^2$. Where indicated, each of the R and S groups were randomized to one of three treatment groups by a Latin square procedure according to the short-circuit current (I_{sc}).

After randomization, monolayers were exposed to MC, GC, or vehicle (control, ethanol) for 24 hr. GC consisted of 100 nM dexamethasone and 10 μM of the mineralocorticoid receptor antagonist spironolactone. MC consisted of 100 nM aldosterone and 10 μM of the glucocorticoid receptor antagonist RU 38486 [45]. These treatments provide nearly complete occupancy of the glucocorticoid and mineralocorticoid receptors, respectively, with minimal crossover occupancy [18].

Electrical Measurements

Measurements of R_T and I_{sc} were initially made under sterile conditions by placing the filter-bottom cylinders into modified Ussing chambers (Jim's Instruments, Iowa City, IA). Measurements were made in media without additives at 37°C using a University of Iowa voltage clamp [17, 18, 24]. A positive I_{sc} indicates a flow of positive charges from apical to basal surface. Electrical measurements for pump current were made in nonsterile chambers designed to accommodate Millicell PCF filters, as described [20].

The solution used to determine the apparent K_m for ouabain contained (in mM) 25 NaCl, 120 K gluconate, 5 Na HEPES, 5 acid HEPES, 1.5 $CaCl_2$, 1 $MgCl_2$, 5 $BaCl_2$, and 5 D-glucose, pH 7.4 and was continuously gassed with air. Ba^{2+} was included to block any residual K^+ flux through K^+ channels on the basolateral membrane. Pilot experiments have shown that 5 mM Ba^{2+} added to the basolateral membrane inhibits I_{sc} in intact monolayers, reflecting the presence of Ba^{2+}-sensitive K^+channels. Amphotericin B (30 μM) was added to the apical solution to permeabilize the apical membrane to monovalent ions. Ouabain was dissolved as a stock and added to the bathing solutions to achieve increasing concentrations.

The initial solution used to determine the apparent K_m of the Na/K pump for Na^+ contained (in mM) 90 K gluconate, 25 tetramethyl ammonium (TMA) Cl, 35 TMA OH, 30 gluconic acid, 10 acid HEPES, 1.5 $CaCl_2$, 1 $MgCl_2$, 5 $BaCl_2$, and 5 D-glucose, pH 7.4. The initial solution used to determine the apparent K_m of the Na/K pump for K^+ contained (in mM) 30 Na gluconate, 25 NaCl, 90 TMA OH, 90 gluconic acid, 5 Na HEPES, 5 acid HEPES, 1.5 $CaCl_2$, 1.0 $MgCl_2$, 5 $BaCl_2$, and 5 D-glucose, pH 7.4. After addition of 30 μM amphotericin B, the $[Na^+]$ or the $[K^+]$ was increased symmetrically by replacing a portion of the bathing solutions with one where Na^+ or K^+ replaced TMA^+ so that the concentrations of all other components were constant. After each replacement, samples of the solutions were measured for $[Na^+]$ and $[K^+]$ by flame photometry.

The basolateral K^+ permeability was determined in S and R monolayers treated with or without 100 nM dexamethasone and aldosterone. The solution used to bathe the basolateral surface contained (in mM): 1.5 $CaCl_2$, 1 $MgCl_2$, 5 Na HEPES, 5 acid HEPES, 25 NaCl, 5 glucose, 120 Na gluconate, and 1 mM ouabain. The apical solution contained (in mM): 1.5 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 10 acid HEPES, 5 KOH, 25 KCl, 120 K gluconate, and no ouabain. Amphotericin B (30 μM) was added to the apical solution, the increase in I_{sc} recorded, and when the current was stable, 10 mM Ba^{2+} was added to the solutions. This concentration of Ba^{2+} inhibited the I_{sc} to the pre-amphotericin value and was therefore taken as a measure of the basolateral K^+ conductance.

Ouabain Binding

Ouabain binding was performed as previously described in this laboratory [24] and is a modification of the method developed for isolated nephron segments [7]. Intact filter cups containing the confluent IMCD monolayer were incubated at 37°C for 1 hr in one of two ^{3}H-ouabain-containing (100 μCi/ml) Hepes buffer solutions. The first solution was K^+ free to determine total ouabain binding and contained (in mM): 1.8 $CaCl_2$, 0.8 $MgSO_4$, 1.0 NaH_2PO_4, 10 Na HEPES, 10 H HEPES, 7.8 glucose, 1 Na pyruvate, and 120 NaCl. The second solution (used to estimate nonspecific binding) was modified to contain 30 mM KCl (replacing NaCl) and 2 mM unlabeled ouabain. After the incubation, monolayers were rinsed as described [24], the filters cut from the plastic rings, and incubated in 0.2% SDS overnight. An aliquot was removed for protein determination and the remainder was counted in a liquid scintillation counter. Specific ouabain binding was calculated as the difference between average values of total and nonspecific binding measured in groups of monolayers.

Quantitative Analysis

Values are reported as mean ± SEM. Statistical analysis was performed using Student's *t* test or analysis of variance (ANOV) with subsequent application of the Newman-Keuls or Bonferroni test as appropriate. Significance was concluded when $P < 0.05$. Nonlinear regression analysis for calculating kinetic constants was conducted using the program Slide Write Plus.

Materials

Chemicals were purchased from Sigma (St. Louis, MO) or Fisher (Pittsburgh, PA) unless otherwise indicated. RU 38486 was a generous gift from Roussel Uclaf, Romainville, France. Benzamil was obtained from Merck (West Point, PA), collagenase from Boehringer Mannheim Diagnostics (Indianapolis, IN), albumin from Intergen (Purchase, NY) and SDS from Bio-Rad Laboratories (Richmond, CA). Gentamicin and other tissue culture materials were purchased from the University of Iowa Cancer Center. Polycarbonate filters were purchased from Poretics (Livermore, CA). Plastic cylinders were purchased from ADAPS (Dunham, MA).

Results

''Back-door'' Phosphorylation of the Na/K Pump

We measured Na/K ATPase activity in 10 membrane fractions from kidney cortex that had been separated by

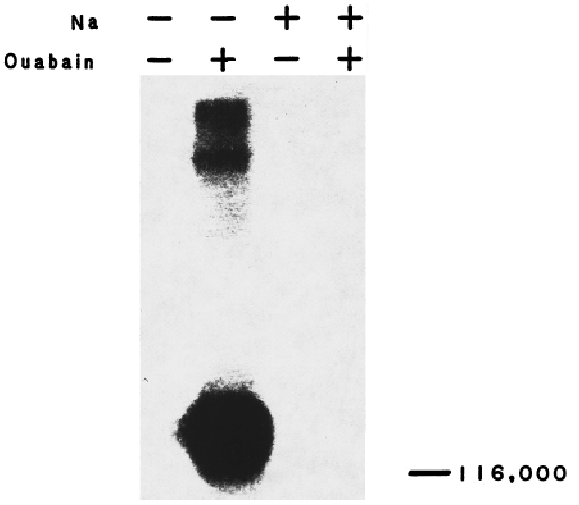

Fig. 1. Autoradiograph of an acid SDS-PAGE after conducting ''back-door'' phosphorylation of the α_1 subunit of the Na/K ATPase [40]. There is greatly reduced phosphorylation in the presence of Na^+ or the absence of ouabain, thus documenting the specificity of the reaction. Higher molecular weight bands may represent multimers of the α_1 subunit.

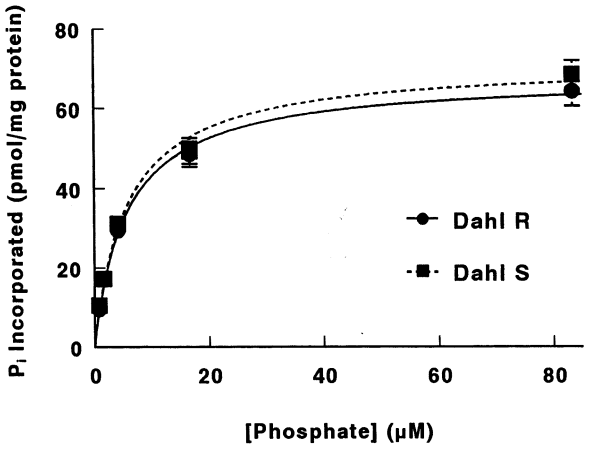

Fig. 2. Incorporation of inorganic phosphate into renal cortical basolateral membranes from Dahl S and R rats. Lines represent nonlinear fit of the data to the Michaelis-Menton equation.

Percoll gradient. The highest activity was found in fractions 3 and 4, while the peak γ-glutamyl transferase activity was found in fractions 8–10. These results are similar to those described by Scalera [44]. We therefore used fractions 3 and 4 for subsequent analysis of basolateral membrane Na/K pumps.

''Back-door'' phosphorylation of Na/K pumps takes advantage of the ability of orthophosphate to become incorporated into the α_1 subunit protein. The phosphorylation occurs on the same aspartyl residue as occurs when the α_1 subunit of the pump is phosphorylated by ATP when running in the ''forward'' direction [40]. ''Back-door'' phosphorylation requires ouabain and the absence of Na^+. Figure 1 shows an acid SDS-PAGE autoradiogram of the reaction products. The phosphorylated band appears only under conditions where ouabain is present and Na^+ is not, thus demonstrating the appropriate criteria for the ''back-door'' reaction. In addition, the phosphorylated protein appears at the appropriate molecular weight for the α_1 subunit of the Na/K ATPase [40].

The incorporation of phosphate into basolateral membranes as a function of concentration, shown in Fig. 2, displayed saturation kinetics. The K_m for phosphate, and the number of pump sites were the same for both S and R membranes (Table 1). The Na/K ATPase activity of these basolateral membranes also was not different (Table 1). Using these values to calculate the turnover number also revealed no differences. Thus, we detected no differences between S and R Na/K pumps in kidney cortex using these approaches.

Table 1. Analysis of Na^+ pumps on basolateral membranes of Dahl rats by ''backdoor'' phosphorylation

	Dahl R	Dahl S
Na-K ATPase activity (nmol/mg protein/min)	892 ± 41	919 ± 36
K_m for phosphate (μM)	5.73 ± 0.73	5.77 ± 0.53
Na^+ pump sites (pmole/mg protein)	67.7 ± 3.8	71.1 ± 4.2
Turnover number for ATP* (#/site/min)	13,300 ± 400	13,100 ± 600

Values represent mean ± SEM from 7 preparations of each strain. There are no differences between Dahl S and R rats. *Values calculated from Na/K ATPase activity and number of pump sites from backdoor phosphorylation.

Cultured IMCD Cells: Na^+ Pump Kinetics and Basolateral K^+ Conductance

To analyze the basolateral membrane transport components of intact IMCD cells, we used the technique of permeablizing the apical membrane with amphotericin B [22]. We first determined the optimal conditions. The effect of increasing the concentration of amphotericin B (in the apical solution) on pump current was the same in Dahl S and R monolayers; concentrations above 10 μM produced no further increase in ouabain-sensitive I_{sc}. Therefore we used 30 μM amphotericin B for all subsequent experiments. Under these conditions, addition of Ba^{2+} to the basolateral solution had no detectable effect on pump current, although it did inhibit I_{sc} in the absence of amphotericin B (*data not shown*). These results suggest that in the presence of apical amphotericin B there is little or no K^+ recycling across the basolateral membrane which could influence the magnitude of the pump current.

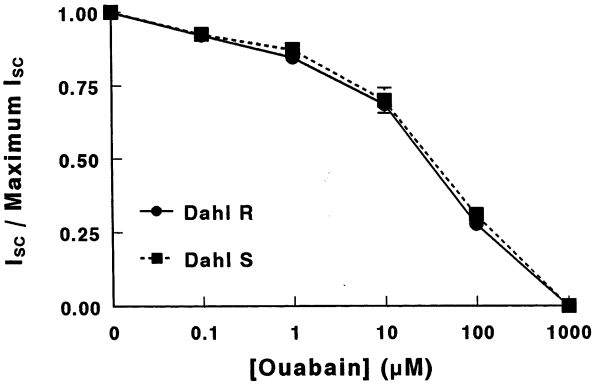

Fig. 3. Inhibition of Na/K pump current by ouabain. Apical membranes of IMCD cells cultured on filters were treated with 30 μM amphotericin B as described in Materials and Methods. Data expressed as fractional inhibition of I_{sc}. Half-maximal inhibition occurred at 55.0 ± 5.5 μM for R monolayers and 57.8 ± 9.5 μM for S monolayers; values are not different. $n = 8$ monolayers from 2 isolations for each group.

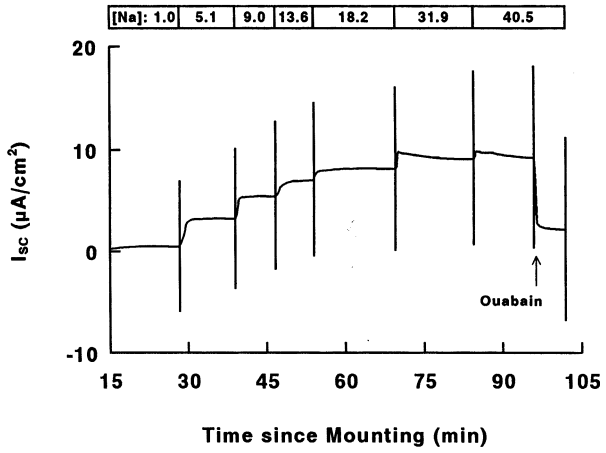

Fig. 4. Example of short circuit current (I_{sc}) response to increasing [Na^+] in the apical and basolateral solutions of IMCD cells. Apical solution contained 30 μM amphotericin B. Vertical lines represent current pulses to ±1 mV. Measured [Na^+] is indicated in the bar over the graph. Solutions were changed so that only the [Na^+] and [TMA] were varied.

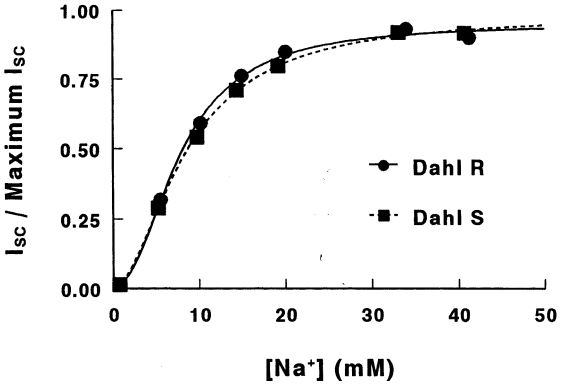

Fig. 5. Response of the Na/K pump to [Na^+]. Values represent mean ± SEM of IMCD cells from 16 R monolayers and 15 S monolayers from 3 isolations. Monolayers were treated with GC. Virtually identical results were obtained with MC treatment (*not shown*). Apical solution contained 30 μM amphotericin B.

The effect of ouabain concentration on pump current is shown in Fig. 3. There are 3 important points. First, the I_{sc} was relatively resistant to ouabain, with K_i values greater than 50 μM. Second, the K_i values for S and R monolayers were not different. Third, the fit of the curve did not improve when we included the option of two binding sites with different affinities. We cannot eliminate the possibility that a small number of pumps are highly sensitive to ouabain. However, these results suggest that the Na/K pump of both the S and R IMCD monolayers is primarily of the α_1 type [28, 29].

We next determined the apparent affinity of the pump for Na^+. Figure 4 demonstrates a typical example of the effect of replacing the TMA solution with one containing Na^+ on I_{sc}. Also shown is the effect of ouabain at the completion of the experiment. Figure 5 shows the data for S and R IMCD monolayers treated with GC for 24 hr. The results using monolayers treated with MC were indistinguishable. The curve and the computed kinetic constants (Table 2) were in general agreement with those reported by other investigators using other epithelial cells and similar approaches [10, 27]. The [Na^+] providing half maximal stimulation of the pump was ~8 mM and was not different between S and R monolayers.

The results of similar experiments examining the apparent affinity of the pump for K^+ are shown in Fig. 6. In these monolayers, where [Na^+] was 60 mM, the apparent affinity for K^+ and the Hill coefficients for S and R monolayers were not different. The computed kinetic constants are shown in Table 2.

We also analyzed the basolateral K^+ conductance of IMCD monolayers from S and R rats by imposing an apical-to-basolateral K^+ gradient and measuring the Ba^{2+}-sensitive I_{sc}. Table 3 shows that there is no difference between S and R monolayers and that treatment with steroids does not affect the magnitude of this current. These results are the same whether the values are factored by area or by protein. The magnitude of the I_{sc} measured in these same monolayers before these K^+ conductance values were determined was similar to that which we have previously reported [19], indicating that the steroids had their typical effect and that Na^+ transport by S monolayers was substantially greater than R monolayers. We note that these results are somewhat different from those reported for A6 cells and TBM cells [3]. The reason for this difference is unclear. However, the important conclusion is that we can detect no difference in basolateral K^+ conductance between S and R monolayers.

Table 2. Kinetic characteristics of the Na/K pump of IMCD monolayers from Dahl S and R rats

	Dahl R	Dahl S
Na^+ kinetics		
(F;I)	(32;4)	(27;4)
Apparent K_m (mM)	8.49 ± 0.31	8.33 ± 0.46
Hill coefficient	2.21 ± 0.13	2.10 ± 0.11
K^+ kinetics		
(F;I)	(18;3)	(17;3)
Apparent K_m (mM)	0.60 ± 0.05	0.57 ± 0.04
Hill coefficient	1.47 ± 0.14	1.47 ± 0.14

Number of filters (F) and isolations (I). Values were computed using the best fit to the equation $I_{sc} = (I_{sc}^{max} - I_{sc}^{min})/(1 + (K_m/[X])^n) + I_{sc}^{min}$ where I_{sc}^{max} and I_{sc}^{min} are the maximal and minimum values of the I_{sc} for all concentrations and $[X]$ is the concentration of either Na^+ or K^+. There are no differences between S and R monolayers.

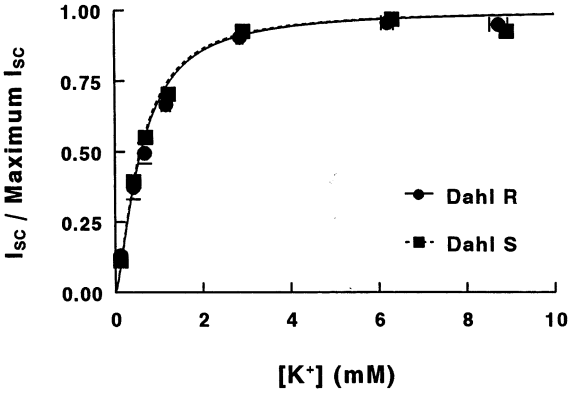

Fig. 6. Response of the Na/K pump to $[K^+]$. Values represent mean ± SEM of 17 R monolayers and 18 S monolayers from 3 isolations. Monolayers treated with aldosterone and dexamethasone. Apical solution contained 30 μM amphotericin B. $[Na^+]$ was maintained at 60 mM bilaterally.

Maximum Pump Capacity and Number of Na^+ Pumps

In the steady state, the rate of Na^+ transport across the apical membrane (through the Na^+ channel) must be equal to the rate of exit through the basolateral membrane Na^+ pump. We analyzed each pathway by measuring the steady state I_{sc} and then the maximum pump current after permeabilizing the apical membrane with amphotericin B. Table 4 shows the values for the steady-state current in intact IMCD cells and the pump currents for the same monolayers. There are four important points. First, S monolayers had larger I_{sc} values than R monolayers. Second, both GC and MC stimulated the I_{sc} in both S and R monolayers; the fractional stimulation was similar, but the absolute increase was larger in S monolayers. These results are consistent with those we have previously reported for S and R monolayers [19].

Table 3. Evaluation of basolateral membrane K^+ conductance in S and R IMCD monolayers

	Ba^{2+}-sensitive I_{sc} ($\mu A/cm^2$)	
	Control	Steroid
Dahl R	3.85 ± 0.64	4.00 ± 0.85
Dahl S	4.51 ± 0.55	3.20 ± 0.52

$n = 8$ monolayers in each group from 2 isolations. Steroid treatment consisted of 100 nM dexamethasone and 100 nM aldosterone for 24 hr before measurements. I_{sc} was determined by adding 30 μM amphotericin B to the apical solution in the presence of an apical-to-basolateral K^+ gradient as described in Materials and Methods. The (10 mM) Ba^{2+}-sensitive I_{sc} was approximately equal to the increase in I_{sc} following amphotericin B. There was no difference between strains nor was there an effect of steroids (by 2-way ANOV). Protein content (*not shown*) was not different between groups.

Table 4. Steady-state and pump currents for IMCD monolayers from Dahl S and R rats

Monolayer; treatment	(F;I)	I_{sc}	I_{pump}
R; control	46;11	3.35 ± 0.33	6.23 ± 0.38
R; GC	45;11	8.37 ± 0.92*	8.87 ± 0.62*
R; MC	34;8	6.62 ± 1.12*	7.55 ± 0.88*
S; control	47;11	5.57 ± 0.54§	7.93 ± 0.58§
S; GC	44;11	14.58 ± 1.13*§	11.63 ± 0.67*§
S; MC	45;11	17.03 ± 1.41*§	11.17 ± 0.76*§

Monolayers treated with glucocorticoid (GC), or mineralocorticoid (MC), or no steroid (control) for 24 hr as described in Materials and Methods. *F;I*, number of filters and isolations; I_{sc}, steady-state short-circuit current measured in isotonic culture medium; I_{pump}, pump current measured after 30 μM amphotericin B in apical solution. Values in $\mu A/cm^2$. *, greater than control value for the same strain; §, greater than comparably treated group from R monolayers; $P < 0.001$ by ANOV.

Third, the magnitude of the pump current was greater in S than R monolayers by ~35%. Finally, GC and MC increased the pump current in both S and R monolayers by ~38%.

We next computed the fraction of the maximal capacity at which the pump operated in intact, short-circuited monolayers. We assumed that the stoichiometry of the pump was $3Na^+$ to $2K^+$ (vide infra). Thus, this value is the I_{sc} divided by 3 times the pump current [22]. As shown in Fig. 7, two important points emerge. First, either GC or MC treatment increased the fraction of maximal capacity in both S and R monolayers. Second, for any treatment group Na/K pumps in S monolayers always operated at a higher fraction of maximal capacity than pumps in R monolayers.

At least two possibilities could explain the higher

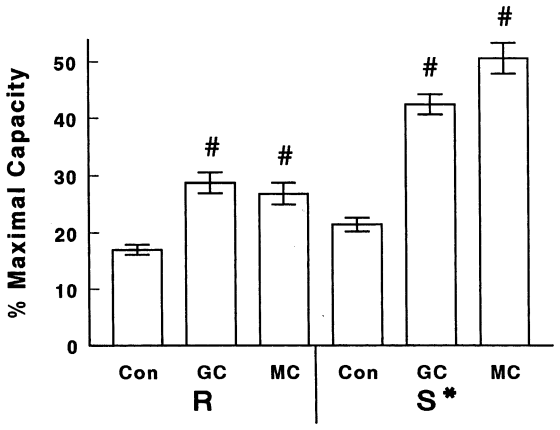

Fig. 7. Fraction of maximal capacity at which the Na/K pump operates in S and R IMCD monolayers. Values computed from data in Table 4 according to the formula $I_{sc}/(I_{pump} \times 3)$. GC, glucocorticoid treated; MC, mineralocorticoid treated. *, S monolayers operate at a greater fraction of their maximum capacity than do R monolayers for any treatment group. #, GC and MC increase the % maximum capacity for both S and R monolayers (by 2-way ANOV).

pump currents in S monolayers (Table 4). First, S monolayers could have more Na/K pumps on the basolateral membrane. A second possibility is that S monolayers have a larger number of cells per monolayer. We have previously shown that S monolayers have, on average, 20% more protein and DNA than R monolayers seeded and grown under these conditions [19]. To evaluate these possibilities, we conducted ouabain binding experiments on S and R monolayers.

As shown in Table 5, Na^+ transport in S monolayers was substantially greater than R monolayers when factored for protein (or area, *not shown*). Despite the different rates of Na^+ transport, there was no difference in specific ouabain binding. These results make it unlikely that the larger pump current in S monolayers is the result of more pumps on the basolateral membrane.

Discussion

The present results confirm that IMCD monolayers cultured from Dahl S rats transport twice as much Na^+ as do IMCD monolayers from R rats ([19]), Table 4). Our analysis of the Na/K pumps from the proximal tubule uncovers no difference between the two strains using backdoor phosphorylation (Table 1). In addition, there are no kinetic differences between Na/K pumps operating in the basolateral membrane of cultured IMCD cells; the apparent affinity for ouabain (Fig. 3), Na^+, or K^+ (Table 2) is the same in S and R monolayers. Finally, the basolateral membrane K^+ conductance is not different between S and R IMCD monolayers (Table 3).

What is the basis for the difference in Na^+ transport?

Table 5. Ouabain binding in Dahl S and R IMCD monolayers

	Dahl R	Dahl S	*P*
F;I	111;5	119;5	
I_{sc} (μA/mg protein)	46.2 ± 3.8	101.7 ± 5.3	<0.001
Spec. binding (cpm/μg protein)	14.3 ± 1.4	15.7 ± 1.5	NS

All monolayers treated with *MC*. F, number of filters; *I*, number of isolations. *P* value by paired analysis; NS, not significant.

We considered the possibility that there are more functioning pumps on the basolateral membrane of S monolayers. The fact that ouabain binding is the same in S and R monolayers (Table 5) suggests that the number of functional pumps is similar. It is unlikely that the equivalent ouabain binding is a result of differing affinities of the pump for ouabain because the K_i for ouabain is the same in S and R monolayers (Fig. 3). In a previous study [24], we easily detected a difference in ouabain binding in IMCD monolayers with different rates of Na^+ transport produced by steroid hormones. Thus, if there were significant differences in the number of pumps on the basolateral membrane accounting for the difference in Na^+ transport in S and R monolayers, we should have detected it.

The fact that the pump current is ~35% larger in S monolayers (Table 4) could be attributed to either (i) a larger number of cells, (ii) a greater capacity per pump, or (iii) a somewhat greater reserve that is unmasked by acutely increasing the Na^+ transport rate with amphotericin B. This latter possibility might be the result of pumps residing in intracellular vesicles that insert into the basolateral membrane when cell $[Na^+]$ is increased. These possibilities, of course, are not mutually exclusive. In any event, the magnitude of the difference in pump currents in S and R monolayers is modest. Our major conclusion from these results is that the increased rate of Na^+ transport by the S monolayers is probably not the result of a primary abnormality of the Na/K pump.

Our conclusion that the Na/K pump of Dahl S rats is not fundamentally different from Dahl R rats contrasts with that of Canessa et al. [4]. Using measurements of ouabain-sensitive Na^+ and Rb^+ fluxes in red blood cells, they found a difference in the apparent K_m for extracellular K^+; Dahl R red blood cells had a substantially higher value (lower affinity) than Dahl S red blood cells. These investigators concluded that the Na:K coupling ratio of the Na/K pump from S rats was 3:1 rather than 3:2, as it appeared to be in Dahl R red blood cells. The reasons for the difference between their conclusions and ours are not immediately obvious, but we offer the following observations. First, implicit in their reasoning is the assumption that ouabain does not secondarily alter

another Na^+ or K^+ membrane transporter(s). This assumption is difficult to ascertain and, at least in some cells, appears to be unwarranted [5]. Second, another study on the kinetics of Dahl S and R red blood cell Na/K pumps failed to detect appreciable differences [55]. Third, a study by Nishi et al. [31] using isolated membranes from Dahl S and R kidneys found no difference in Na/K pump kinetics and apparent affinity for K^+. However, this same study did find some differences in Na/K pump kinetics when intact proximal tubule cells were used.

There is some evidence that the Dahl S Na/K pump can operate differently than the Dahl R Na/K pump in intact cells. In addition to the previously mentioned differences reported by Canessa et al. [4], Nishi et al. [33] have reported that the Na/K pump activity of proximal tubule cells from Dahl S rats is less responsive to dopamine than that of Dahl R rats. This group has also documented that a high NaCl diet increases Na/K pump activity in Dahl S proximal tubules but not in Dahl R proximal tubules [32]. Distal tubular cells from S rats respond differently to partial inhibition of their Na/K pump than do those from R rats [38]. Other workers have documented that differences in Na/K pump activity between S and R rats are not confined to the kidney; vascular tissue can also show differences [35]. Age, dietary salt, and blood pressure have all been implicated in modifying the Na/K pump activity in intact cells [14, 55]. Thus, despite the evidence that the Na/K pump might play an important role in the pathogenesis of hypertension in Dahl S rats, the specific molecular nature of its role remains unclear.

Because the magnitude of the difference in the pump current could not fully account for the difference in Na^+ transport by intact S and R IMCD monolayers, we calculated the fraction of the maximal capacity at which the Na/K pump operates. To compute the maximal rate of Na^+ transport through the pump, we assumed that the stoichiometry was $3Na^+$:$2K^+$, an assumption with considerable experimental support. Although we have not measured the pump stoichiometry in S and R IMCD monolayers, the present data support the conclusion that the stoichiometry of the Na/K pumps from S and R monolayers is the same; there is no difference in the affinities for Na^+ or K^+ and the Hill coefficients are indistinguishable (Table 2).

In intact IMCD cells, K^+ entering the cell via the Na/K pump exits across the basolateral membrane [18]. In amphotericin B treated cells, K^+ exits across the apical membrane [22]. Thus the I_{sc} of monolayers treated with amphotericin B represents ⅓ of the Na^+ translocated by the pumps across the basolateral membrane if the overall stoichiometry is $3Na^+$:$2K^+$ [22]. The same conclusion can be made even if the Na/K stoichiometry were not 3:2. The only requirements are that the overall charge stoichiometry is 3:2 and that the cations entering the cell via the pump exit across the apical membrane in amphotericin B treated cells. For example, if the counter ion were H^+ instead of K^+, the relationship between pump Na^+ transport and pump current in amphotericin B-treated monolayers would be the same so long as H^+ exit was predominantly across the apical membrane. There is excellent experimental evidence indicating that such is the case in rabbit urinary bladder [6]. We will therefore proceed with the analysis assuming a pump (charge) stoichiometry of 3:2.

From the data in Table 4, it is apparent that the Na/K pumps of IMCD monolayers from Dahl S rats operate at a higher fraction of their maximal capacity than do pumps from Dahl R rats (Fig. 7). The only known explanation for this situation is that the steady-state intracellular $[Na^+]$ is higher in IMCD cells from S rats than comparable cells from R rats. The relationship between IMCD cell $[Na^+]$ and pump current is shown in Fig. 5 and allows us to compute the following quantitative specifics. For the Na/K pump to increase its fractional capacity from 20% to 50%, the cell $[Na^+]$ would increase from ~5 mM to ~8 mM. This difference is sufficiently small that measurements of cell $[Na^+]$ in S and R IMCD monolayers would not likely detect a significant difference.

The deduction that intracellular $[Na^+]$ is higher in S monolayers than R monolayers leads naturally to the question of how this situation might occur. The most likely explanation is that the apical membrane Na^+ channels of S monolayers permit more Na^+ to enter than do Na^+ channels of R monolayers. This explanation is consistent with the generally held notion that the apical membrane entry pathway is usually rate limiting for transepithelial Na^+ transport. These results thus point to the epithelial Na^+ channel as a candidate molecule for being abnormal and/or abnormally regulated in salt-sensitive hypertension, at least in this strain of Dahl rat.

The possibility that one or more of the subunits of the epithelial Na^+ channel might be abnormal in Dahl S rats is made even more plausible by the recent observation that a familial form of hypertension in humans, Liddle's Syndrome, is caused by sequence abnormalities of the β- and γ-subunits [11, 13, 46, 49]. We have recently determined that the Na^+ channels comprising the predominant pathway in apical membranes of Wistar rat IMCD cells is the equivalent of the human epithelial Na^+ channel, ENaC [53]. The extent to which this complex participates in Na^+ transport by Dahl S and R IMCD monolayers has not yet been ascertained.

Could any of the epithelial Na^+ channel subunits be abnormal in Dahl rats? The relevant information to date can be summarized as follows. The β and γ subunits are located on chromosome 1 of the rat and both are close to the Sa gene [16]. The Sa gene locus cosegregates with hypertension in the second generation of Dahl S rats

crossed with the Lewis strain [9]. It is not known whether the Sa gene itself, or a nearby gene, participates in the genetic predisposition to hypertension. The Sa gene is overexpressed in Dahl S kidneys, but its function is unknown [12]. Finally, the published data on the β subunit indicates that there is no sequence difference between S and R rats [16]. Obviously, understanding the precise role of the epithelial Na^+ channel subunits in the pathogenesis of salt-sensitive hypertension will require more investigation.

Our integrated assessment of the data presented in this manuscript is that an abnormality of the Dahl S (or R) IMCD cell function could play an important role in the pathophysiology of salt-sensitive hypertension. The specific derangement is likely to involve the apical membrane Na^+ channel subunits or a molecule(s) involved in their regulation.

We appreciate the technical assistance of Bonnie Moses and Rita Sigmund and the assistance of Dick Shaffer for breeding the rats. This research was supported in part by National Institutes of Health grants, HL55006, HL44546, and a grant from the Department of Veterans Affairs.

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