

Hyposmolality stimulates apical membrane Na^+/H^+ exchange and HCO_3^- absorption in renal thick ascending limb

Bruns A. Watts III and David W. Good

Department of Medicine and Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77555, USA

Address correspondence to: David W. Good, 4.200 John Sealy Annex 0562, University of Texas Medical Branch, 301 University Boulevard, Galveston, Texas 77555-0562, USA.

Phone: (409) 772-2472; Fax: (409) 772-5451; E-mail: dgood@utmb.edu.

Received for publication May 13, 1999, and accepted in revised form October 12, 1999.

The regulation of epithelial Na^+/H^+ exchangers (NHEs) by hyposmolality is poorly understood. In the renal medullary thick ascending limb (MTAL), transepithelial bicarbonate (HCO_3^-) absorption is mediated by apical membrane Na^+/H^+ exchange, attributable to NHE3. In the present study we examined the effects of hyposmolality on apical Na^+/H^+ exchange activity and HCO_3^- absorption in the MTAL of the rat. In MTAL perfused in vitro with 25 mM HCO_3^- solutions, decreasing osmolality in the lumen and bath by removal of either mannitol or sodium chloride significantly increased HCO_3^- absorption. The responses to lumen addition of the inhibitors ethylisopropyl amiloride, amiloride, or HOE 694 are consistent with hyposmotic stimulation of apical NHE3 activity and provide no evidence for a role for apical NHE2 in HCO_3^- absorption. Hyposmolality increased apical Na^+/H^+ exchange activity over the pH_i range 6.5–7.5 due to an increase in V_{max} . Pretreatment with either tyrosine kinase inhibitors or with the tyrosine phosphatase inhibitor molybdate completely blocked stimulation of HCO_3^- absorption by hyposmolality. These results demonstrate that hyposmolality increases HCO_3^- absorption in the MTAL through a novel stimulation of apical membrane Na^+/H^+ exchange and provide the first evidence that NHE3 is regulated by hyposmotic stress. Stimulation of apical Na^+/H^+ exchange activity in renal cells by a decrease in osmolality may contribute to such pathophysiological processes as urine acidification by diuretics, diuretic resistance, and renal sodium retention in edematous states.

J. Clin. Invest. **104**:1593–1602 (1999).

Introduction

Na^+/H^+ exchange is present in the plasma membrane of virtually all cells and plays a major role in a variety of cell functions, including control of cell volume, regulation of intracellular pH (pH_i), reabsorption of sodium chloride (NaCl) and sodium bicarbonate (NaHCO_3) by epithelial cells and proliferation (1–3). At least 5 mammalian isoforms of Na^+/H^+ exchange (NHE1–NHE5) have been identified (3–5). These isoforms differ in their tissue distribution, sensitivity to amiloride analogs, and responses to physiological stimuli (2–5).

A prominent feature of Na^+/H^+ exchangers (NHEs) is their regulation by osmotic stress. In many cells, Na^+/H^+ exchange activity is increased in response to hyperosmotic cell shrinkage, resulting in increased cellular uptake of NaCl and H_2O that returns cell volume toward its original value (1, 6). This volume regulatory response generally is mediated through hyperosmotic activation of NHE1, the ubiquitously expressed exchanger isoform present on the plasma membrane of nonpolar cells and the basolateral membrane of epithelial cells (3–5, 7, 8). Hyperosmolality also influences the activities of NHE2, NHE3, and NHE4, isoforms that exhibit a more restricted tissue distribution, with pref-

erential localization in epithelial cells of the kidney and gastrointestinal tract (3–5, 9). Similar to NHE1, NHE2 and NHE4 are activated by hyperosmolality and may play a role in the regulation of cell volume (9–11). In contrast, hyperosmolality inhibits NHE3 (10, 12–15). This isoform is located predominantly in the apical membrane of renal tubule and intestinal epithelial cells, where it plays a major role in mediating the transepithelial reabsorption of NaCl and NaHCO_3 (3, 5, 13, 16–20). Thus, NHE isoforms are regulated differentially by hyperosmolality, probably reflecting the fact that the isoforms subserve different physiologic functions, such as cell volume regulation and transepithelial sodium reabsorption, that may need to be regulated independently in response to hyperosmotic stress.

In contrast to hyperosmolality, the effects of hyposmolality on NHEs have not been clearly defined. Hyposmolality has been shown to decrease Na^+/H^+ exchange activity in several systems (10, 21–23), effects that are attributable to hyposmotic inhibition of the housekeeping isoform NHE1 (10, 22, 23). The physiologic responses of other isoforms to hyposmotic stress are not understood. In a study of epithelial isoforms expressed in an NHE-deficient cell line, hyposmolality

inhibited NHE2 activity but had no effect on NHE3 activity (10). Hyposmolality also had no effect on NHE3 activity in LLC-PK₁ cells, a renal epithelial cell line (23). The latter observations suggest that hyposmolality may not be involved in the physiological regulation of NHE3 and its primary function, transepithelial sodium absorption. However, no studies have examined the effects of hyposmolality on NHE3 activity in intact, native epithelia. Thus, it is unclear whether NHE3 is physiologically unresponsive to hypotonic stress or whether its regulation by hyposmolality may be cell-type specific, requiring regulatory elements that are present in native epithelial cells but lacking in NHE-transfected cells or established cell lines.

The medullary thick ascending limb (MTAL) of the mammalian kidney participates in the regulation of acid-base balance by reabsorbing most of the filtered HCO₃⁻ that is not reabsorbed by the proximal tubule (24). The H⁺ secretion necessary for this HCO₃⁻ absorption is mediated virtually completely by apical membrane Na⁺/H⁺ exchange (18) attributable to NHE3 (13, 16, 20, 25). Because of its location in the renal medulla, the MTAL is routinely exposed to rapid and large variations in lumen and interstitial osmolality during changes in H₂O balance due to the operation of the urinary concentrating mechanism (26). Recently, we demonstrated that peritubular hyperosmolality markedly inhibits HCO₃⁻ absorption in the MTAL through inhibition of apical membrane Na⁺/H⁺ exchange (NHE3) activity (12, 13). This inhibition was mediated through a tyrosine kinase-dependent signaling pathway and was due to a decrease in the apparent affinity of the exchanger for intracellular H⁺ (12, 13). These findings established that osmolality is an important determinant of the rate of luminal acid secretion and HCO₃⁻ absorption by renal tubule segments. At present, however, the effects of hyposmolality on Na⁺/H⁺ exchange activity and HCO₃⁻ absorption in renal tubules are largely unknown. The signal transduction pathways that mediate hypotonic regulation of Na⁺/H⁺ exchange activity also have not been identified.

The purpose of the present study was to examine the effects of hyposmolality on apical membrane Na⁺/H⁺ exchange activity and HCO₃⁻ absorption in the MTAL of the rat. The results demonstrate that peritubular hyposmolality increases HCO₃⁻ absorption through a novel stimulation of apical membrane Na⁺/H⁺ exchange activity, attributable to NHE3. The hypotonic stimulation of HCO₃⁻ absorption is mediated through a tyrosine kinase-dependent signaling pathway. Stimulation of apical membrane Na⁺/H⁺ exchange activity in renal tubule cells by a decrease in osmolality may contribute to changes in urinary net acid and sodium excretion in a variety of pathophysiological conditions.

Methods

Tubule perfusion. MTAL from male Sprague-Dawley rats (60–90 g; Taconic Farms, Germantown, New York,

USA) were isolated and perfused in vitro as described previously (12, 13, 27, 28). In brief, the tubules were dissected from the inner stripe of the outer medulla, transferred to a bath chamber on the stage of an inverted microscope, and mounted on concentric glass micropipets for perfusion at 37°C. The composition of the perfusion and bath solutions for specific protocols is given below. Solutions containing furosemide, 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), amiloride, *N*-methyl-D-glucammonium (NMDG⁺), 2',7'-bis-(carboxyethyl)-5,6-carboxy-fluorescein (BCECF), and tyrosine kinase inhibitors were prepared as described previously (12, 13). HOE 694 was a gift of Hoechst Pharmaceuticals and was prepared as a 10 mM stock solution in H₂O.

Measurement of net HCO₃⁻ absorption. In experiments in which transepithelial HCO₃⁻ absorption was studied, tubules were perfused and bathed in control solution that contained (in mM) 121 Na⁺, 4 K⁺, 97 Cl⁻, 25 HCO₃⁻, 2.0 Ca²⁺, 1.5 Mg²⁺, 2.0 phosphate, 1.2 SO₄²⁻, 1.0 citrate, 2.0 lactate, 5.5 glucose, and 50 mannitol (osm = 295 mosmol/kg H₂O). Hypotonic solution was identical except for the removal of either 50 mM mannitol (osm = 245 mosmol/kg H₂O) or 25 mM mannitol (osm = 270 mosmol/kg H₂O). In one series of HCO₃⁻ transport experiments (see Figure 1b), 25 mM NaCl replaced 50 mM mannitol in the control perfusate and bath (Na⁺ = 146 mM, Cl⁻ = 122 mM, osmolality = 295 mosmol/kg H₂O). In this series the hypotonic solution was made by the removal of 25 mM NaCl. All solutions were equilibrated with 95% O₂, 5% CO₂ (pH 7.45) at 37°C. Bath solutions also contained 0.2 g/100 mL fatty acid-free BSA. Experimental agents were added to the lumen or bath solutions as described in Results. The tubules were dissected at 10°C in the control solution that contained 146 mM Na⁺ and 122 mM Cl⁻ (see above). The length of the perfused tubule segments ranged from 0.51 to 0.70 mm.

The protocol for study of transepithelial HCO₃⁻ absorption was as described (13, 27). The tubules were equilibrated for 20 to 30 minutes at 37°C in the initial perfusion and bath solutions, and the luminal flow rate (normalized per unit tubule length) was adjusted to 1.4–1.9 nL/min per millimeter. Two or three 10-minute tubule fluid samples were then collected for each period (initial, experimental, and recovery). The tubules were allowed to re-equilibrate for 5 to 15 minutes after a change in the composition of the lumen and/or bath solutions. The absolute rate of HCO₃⁻ absorption (JHCO₃⁻, pmol/min per millimeter) was calculated from the luminal flow rate and the difference between total CO₂ concentrations measured in perfused and collected fluids (13, 27). An average HCO₃⁻ absorption rate was calculated for each period studied in a given tubule. When repeat measurements were made at the beginning and end of an experiment (initial and recovery periods), the values were averaged. Single tubule values are presented in the figures. Mean values ± SE (*n* = number of tubules) are presented in the text.

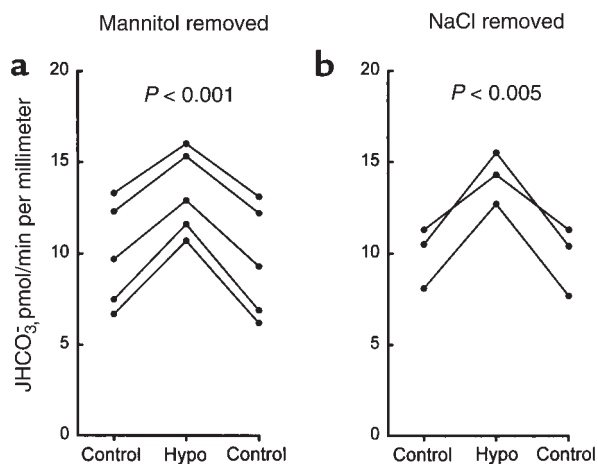


Figure 1

Hyposmolality stimulates HCO_3^- absorption in the MTAL. Osmolality in lumen and bath solutions was decreased from 295 mosmol/kg H_2O (Control) to 245 mosmol/kg H_2O (Hypo) by removal of either 50 mM mannitol (a) or 25 mM NaCl (b) from the control solution. JHCO_3^- , absolute rate of HCO_3^- absorption. Data points are average values for single tubules. Lines connect paired measurements made in the same tubule. P values are for experimental period (Hypo) versus either initial or recovery period (Control) by ANOVA. Mean values are presented in Results.

Measurement of pH_i . The pH_i was measured by using the pH-sensitive dye BCECF and a computer-controlled spectrofluorometer (CM-X; SPEX Industries, Edison, New Jersey, USA) coupled to the perfusion apparatus, as described previously (12, 28, 29). The tubules were perfused in the same manner used for HCO_3^- transport experiments except that the lumen and bath solutions were delivered using rapid-flow systems that permit complete exchange of the solutions in less than 2 seconds (28). Intracellular dye was excited alternately at 500-nm and 440-nm wavelengths and emission was monitored at 530 nm using a photon counter. Intracellular dye was calibrated using high K^+ -nigericin standards at the end of each experiment to convert fluorescence excitation ratios (F_{500}/F_{440}) to pH_i values, as described previously (12, 28). For tubules studied in isosmotic solution, the calibration solution contained (in mM) 95 K^+ , 10 Na^+ , 107 Cl^- , 2.0 Ca^{2+} , 1.5 Mg^{2+} , 2.5 phosphate, 25 HEPES, 5 glucose, 50 mannitol, and 0.01 nigericin (titrated to selected pH values between 6.5 and 7.5). For experiments with hyposmotic solution, calibrations were performed using the same standards made hyposmotic by the removal of 50 mM mannitol. Consistent with previous results (12, 30), decreasing the K^+ concentration of the hyposmotic calibration solutions by an amount inversely proportional to the increase in cell volume (see below) had no effect on excitation ratios. Mean excitation ratios and the mean calibration slope did not differ with isosmotic and hyposmotic calibration solutions (slope = 0.34 ± 0.02 pH unit, isosmotic, versus 0.38 ± 0.02 pH unit, hyposmotic; $P = \text{NS}$).

Measurement of apical membrane Na^+/H^+ exchange activity. Apical membrane Na^+/H^+ exchange activity was determined as described previously (12). The tubules were perfused and bathed in either isosmotic or hyposmotic HEPES-buffered solution. The isosmotic solution contained (in mM) 120 Na^+ , 4 K^+ , 122 Cl^- , 2.0 Ca^{2+} , 1.5 Mg^{2+} , 1.0 phosphate, 1.0 SO_4^{2-} , 1.0 citrate, 2.0 lactate, 5.5 glucose, 5 HEPES, and 50 mannitol. The hyposmotic solution was identical except for the removal of 50 mM mannitol. Both solutions were equilibrated with 100% O_2 and titrated to pH 7.4. The lumen solutions also contained furosemide to block $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transport-mediated changes in cell Na^+ concentration or volume, and the bath solutions contained EIPA to eliminate any contribution of basolateral membrane Na^+/H^+ exchange to changes in pH_i . After a stable pH_i was reached, Na^+ was replaced completely with NMDG $^+$ in the lumen and bath solutions. Bilateral Na^+ replacement unmasks a background acid-loading process that reduces the pH_i to 6.5–6.7 (12). Apical Na^+/H^+ exchange activity was then determined by measurement of the initial rate of pH_i increase after readdition of Na^+ to the tubule lumen. H^+ flux rates were calculated as $(\text{dpH}_i/\text{dt}) \times \beta_i \times V$, where dpH_i/dt is the initial slope of the record of pH_i versus time (pH units/min) measured over the first 4 seconds following an experimental maneuver (12, 28), β_i is the intrinsic intracellular buffering power (mmol/L \times pH unit), and V is cell volume per millimeter tubule length (nL/mm; see below). To determine the pH_i -dependence of apical membrane Na^+/H^+ exchange,

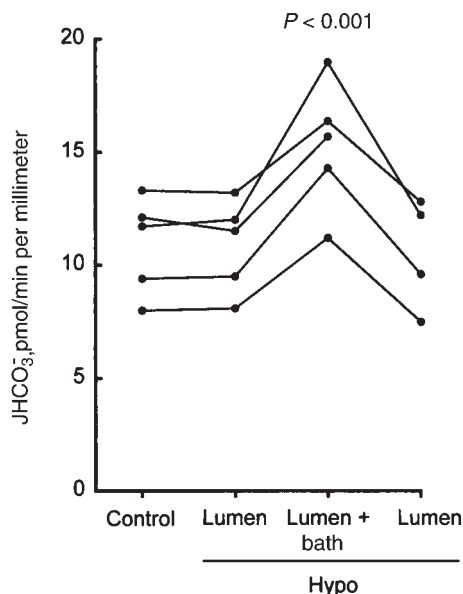


Figure 2

Bath hyposmolality is necessary for stimulation of HCO_3^- absorption. After an initial control period, hyposmolality was produced in the lumen alone (50 mM mannitol removed). The bath was then made hyposmotic in the continued presence of a hyposmotic lumen. JHCO_3^- , data points, and lines as in Figure 1. P value is for lumen plus bath versus either period of lumen alone (ANOVA). $P = \text{NS}$, control versus lumen. Mean values are presented in Results.

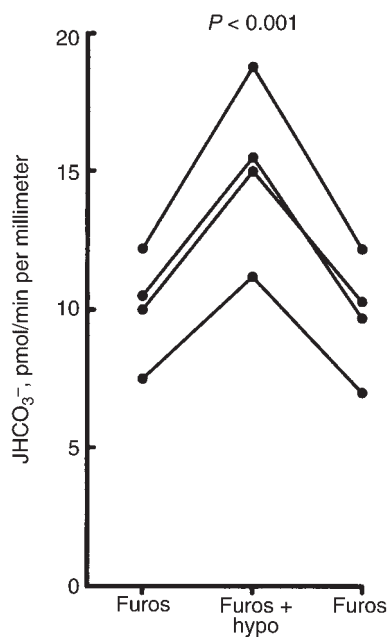


Figure 3

Furosemide has no effect on stimulation of HCO_3^- absorption by hyposmolality. Hyposmolality was produced by removal of 50 mM mannitol from lumen and bath solutions. Furosemide (Furos; 10^{-4} M) was present in the lumen throughout the experiments. JHCO_3^- , data points, lines, and P value are as in Figure 1. Mean values are presented in Results.

the Na^+ -dependent pH_i recovery was interrupted at various points along the recovery curve by luminal Na^+ removal (NMDG⁺) plus EIPA, which unmasks background acid loading (12). At the point of interruption, the Na^+/H^+ exchange rate is calculated as the difference between the net recovery rate and the background acid-loading rate (12, 31). This approach permits the Na^+/H^+ exchange rate to be determined over a range of pH_i values, with appropriate corrections for a variable background acid-loading rate.

To calculate H^+ fluxes, β_i was determined as a function of pH_i using methods described previously (12). The isosmotic solution used to measure β_i was identical to that used previously (12), except 50 mM mannitol replaced 25 mM NMDG gluconate. The hyposmotic solution for determination of β_i was identical to the isosmotic solution, except for the removal of 50 mM mannitol. β_i did not differ in the isosmotic and hyposmotic solutions over the pH_i range studied (6.5–7.6). Similar to previous results (12), β_i decreased linearly with increasing pH_i , averaging 57 ± 2 mM/pH unit at pH_i 6.6 and 39 ± 3 mM/pH unit at pH_i 7.5. V was determined from inner and outer tubule diameters measured under conditions identical to those used for measurement of initial rates of Na^+ -dependent pH_i recovery (12, 13, 32). V was 0.33 ± 0.03 nL/mm ($n = 3$) in isosmotic solution and 0.37 ± 0.03 ($n = 4$) in hyposmotic solution. V did not differ in the absence and presence of lumen Na^+ in either condition. Cell volume also was determined in tubules studied in the control and hyposmotic (245 mosmol/kg H_2O) solu-

tions used in HCO_3^- transport experiments. In these experiments, in which control and hyposmotic measurements were made in the same tubule, V was 0.31 ± 0.02 nL/mm in control solution and increased reversibly to 0.35 ± 0.02 nL/mm in hyposmotic solution ($n = 4$; $P < 0.001$). Thus, in both the pH_i and HCO_3^- transport protocols, hyposmolality induced a small (12–14%) increase in steady-state cell volume.

Statistical analysis. Results are presented as means \pm SE. Differences between means were evaluated using analysis of variance for repeated measurements with Newman-Keuls multiple range test or the Student's t test for unpaired data, as appropriate. P less than 0.05 was considered statistically significant.

Results

Hyposmolality stimulates HCO_3^- absorption. The effects of hyposmolality on transepithelial HCO_3^- absorption are shown in Figure 1. Removal of 50 mM mannitol from the lumen and bath solutions increased HCO_3^- absorption from 9.7 ± 1.3 to 13.3 ± 1.0 pmol/min per millimeter ($n = 5$; $P < 0.001$) (Figure 1a). The stimulation was observed within 15 minutes after mannitol was removed and was reversible. Decreasing osmolality from 295 to 270 mosmol/kg H_2O by the removal of 25 mM mannitol caused a similar stimulation of HCO_3^- absorption (10.3 ± 1.1 pmol/min per millimeter control vs. 12.8 ± 1.2 pmol/min per millimeter hyposmotic, $n = 4$; $P < 0.001$).

To determine whether the stimulation of HCO_3^- absorption was due to the removal of mannitol or to the decrease in osmolality, we examined the effect of removing NaCl. Removal of 25 mM NaCl from the lumen and bath increased HCO_3^- absorption from 9.9 ± 1.0 to 14.2 ± 0.8 pmol/min per millimeter ($n = 3$; $P < 0.005$) (Figure 1b). Thus, the response to NaCl removal was similar to that observed with mannitol removal. These findings indicate that the stimulation of HCO_3^- absorption is due to the decrease in osmolality rather than to the removal of a particular solute or to a change in the solution ionic strength. In subsequent experiments, hyposmolality was produced by the removal of 50 mM mannitol.

Bath hyposmolality is necessary for stimulation of HCO_3^- absorption. Because the thick ascending limb has a very low osmotic water permeability, the reabsorption of sodium salts normally reduces the lumen osmolality to below that of the peritubular fluid (26, 33). We therefore tested whether decreasing the osmolality of the lumen alone would stimulate HCO_3^- absorption. The results in Figure 2 show that removal of mannitol from the tubule lumen only had no effect on HCO_3^- absorption (10.9 ± 0.9 pmol/min per millimeter control vs. 10.8 ± 0.9 pmol/min per millimeter lumen hypo; $n = 5$). Based on previous measurements of osmotic-induced transepithelial water flow in the MTAL (27, 33, 34), the water flux induced by the removal of lumen mannitol would by itself increase the lumen HCO_3^- concentration by less than 2% at a lumen flow rate of 1.7 nL/min per millimeter. Thus, fluid transport has a negligible

influence on the calculated HCO_3^- absorption rates under the conditions of our experiments.

In contrast, in the continued presence of lumen hyposmolality, removal of mannitol from the bath reversibly increased HCO_3^- absorption from 10.8 ± 0.9 to 15.4 ± 1.3 pmol/min per millimeter ($n = 5$; $P < 0.001$) (Figure 2). These results demonstrate that a decrease in peritubular fluid osmolality is necessary for the hyposmotic stimulation of HCO_3^- absorption.

Furosemide has no effect on stimulation of HCO_3^- absorption by hyposmolality. Changes in extracellular tonicity alter the activity of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransport and other transport pathways involved in NaCl absorption by the MTAL (34, 35). To determine whether the stimulation of HCO_3^- absorption may have been the indirect result of an effect on transcellular NaCl transport, we examined the effect of hyposmolality in tubules perfused with furosemide to block NaCl absorption (33). In MTAL studied with 10^{-4} M furosemide in the tubule lumen, removal of 50 mM mannitol from the lumen and bath solutions increased HCO_3^- absorption from 10.0 ± 1.0 to 15.2 ± 1.4 pmol/min per millimeter ($n = 4$; $P < 0.001$) (Figure 3). Thus the stimulation of HCO_3^- absorption by hyposmolality occurs independent of effects on net NaCl absorption.

Lumen EIPA inhibits stimulation of HCO_3^- absorption by hyposmolality. Absorption of HCO_3^- by the MTAL is mediated by apical membrane Na^+/H^+ exchange (18, 24). Therefore, we tested whether the increase in HCO_3^- absorption induced by hyposmolality was mediated by an increase in apical Na^+/H^+ exchange activity. The contribution of apical membrane Na^+/H^+ exchange to HCO_3^- absorption was assessed by the lumen addition of 50 μM EIPA, which inhibits apical Na^+/H^+ exchange activity and HCO_3^- absorption virtually completely in the MTAL (12, 18, 29). The results in Figure 4a show that in MTAL perfused and bathed in hyposmotic solution, addition of 50 μM EIPA to the lumen decreased HCO_3^- absorption from 13.2 ± 1.1 to 1.1 ± 0.1 pmol/min per millimeter ($n = 4$; $P < 0.001$). Thus, virtually all of HCO_3^- absorption in hyposmotic solution is inhibited by lumen EIPA, which suggests that hyposmolality increases HCO_3^- absorption through stimulation of apical membrane Na^+/H^+ exchange.

The apical membrane Na^+/H^+ exchange activity responsible for HCO_3^- absorption in the rat MTAL is mediated by the relatively amiloride-resistant exchanger isoform NHE3 (13, 16, 20, 25, 29). Recent studies suggest, however, that the apical membrane of the MTAL also may contain the amiloride-sensitive isoform NHE2 and that this isoform may contribute to luminal acidification and HCO_3^- absorption (36, 37). We therefore considered whether the stimulation of HCO_3^- absorption by hyposmolality may involve the activation of NHE2. The experiments in Figure 4a do not distinguish between NHE3 and NHE2 because both isoforms are inhibited by 50 μM EIPA. Therefore, additional experiments were performed using 50 μM amiloride or 100 μM HOE 694, compounds that at the

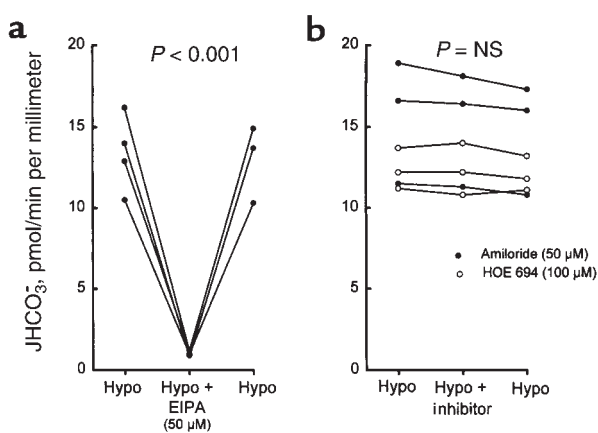


Figure 4

Effects of lumen EIPA, amiloride, and HOE 694 on HCO_3^- absorption in hyposmotic solutions. MTAL were perfused and bathed in hyposmotic solution (50 mM mannitol removed) and then 50 μM EIPA (a) and 50 μM amiloride or 100 μM HOE 694 (b) was added to the tubule lumen. JHCO_3^- , data points, lines, and P values are as in Figure 1. Mean values are presented in Results.

concentrations indicated inhibit NHE2 activity virtually completely but have negligible effects on NHE3 (2, 3). Previously we found that 50 μM lumen amiloride had no effect on HCO_3^- absorption in MTAL studied in control (isosmotic) solutions (29). The results in Figure 4b show that addition of 50 μM amiloride to the lumen also had no effect on HCO_3^- absorption in hyposmotic solution. Lumen addition of 100 μM HOE 694, which has a 2 log-order difference in its inhibitory constant for NHE2 (K_i , 5 μM) versus NHE3 (K_i , 650 μM), also had no effect on HCO_3^- absorption in hyposmotic solution (Figure 4b). For the 6 experiments combined in Figure 4b, the HCO_3^- absorption rate was 13.7 ± 1.1 pmol/min per millimeter for hyposmolality versus 13.9 ± 1.2 pmol/min per millimeter for hyposmolality plus inhibitor ($P = \text{NS}$). Lumen HOE 694 (100 μM) also had no effect on HCO_3^- absorption in control solution (data not shown). Thus, we found no evidence that an amiloride- or HOE 694-sensitive NHE (NHE2) contributes to HCO_3^- absorption under either hyposmotic or isosmotic conditions. Taken together, these findings suggest that the hyposmotic stimulation of HCO_3^- absorption involves stimulation of an amiloride-resistant, HOE 694-insensitive apical membrane Na^+/H^+ exchanger (NHE3).

Hyposmolality stimulates apical membrane Na^+/H^+ exchange activity. Further experiments were carried out to test directly the hypothesis that hyposmolality stimulates apical membrane Na^+/H^+ exchange activity. MTAL were studied in isosmotic or hyposmotic solution, and apical Na^+/H^+ exchange activity was examined directly by measurement of initial rates of pH_i increase after lumen Na^+ addition (see Methods). As shown in Figure 5, hyposmolality increased apical membrane Na^+/H^+ exchange activity over the pH_i range 6.5–7.7. The apical exchanger exhibited a sigmoidal dependence on pH_i , as described

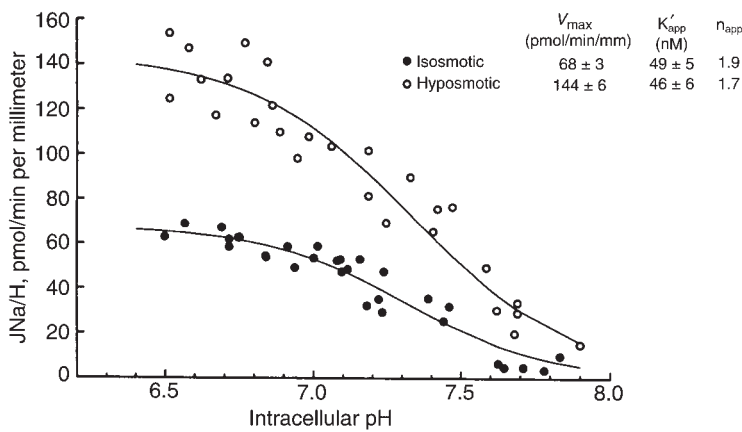


Figure 5

Hyposmolality stimulates apical membrane Na^+/H^+ exchange activity by increasing V_{\max} . MTAL were studied in isosmotic (filled circles) or hyposmotic (50 mM mannitol removed; open circles) solutions. Apical Na^+/H^+ exchange rates ($J_{\text{Na}^+/\text{H}^+}$) were determined at various pH_i values from rates of pH_i recovery measured after addition of Na^+ to the tubule lumen (see Methods). Data points are from 10 tubules in isosmotic solution and 12 tubules in hyposmotic solution. Lines and kinetic parameters are from least-squares fits to the Hill equation.

previously (12, 38). Kinetic analysis showed that the stimulation of Na^+/H^+ exchange activity was due to a 110% increase in V_{\max} (Figure 5). The apparent affinity for intracellular H^+ (K'_{app}) and the apparent Hill coefficient (n_{app}) were similar in isosmotic and hyposmotic solutions. These results demonstrate that hyposmolality stimulates apical membrane Na^+/H^+ exchange activity in the MTAL by increasing its maximal rate of exchange.

To confirm that the Na^+ -dependent pH_i recovery stimulated by hyposmolality was due to Na^+/H^+ exchange, we measured initial rates of pH_i recovery after lumen Na^+ addition in the absence and presence of 50 μM EIPA. The results in Figure 6 show that lumen EIPA inhibited pH_i recovery nearly completely in both isosmotic and hyposmotic solutions. With EIPA in the lumen, the recovery rate was reduced to 3.7 ± 0.8 pmol/min per millimeter ($n = 4$) in isosmotic solution and to 3.6 ± 0.6 pmol/min per millimeter ($n = 4$) in hyposmotic solution (> 90% inhibition). Taken together, our results support the conclusion that hyposmolality stimulates HCO_3^- absorption in the MTAL through stimulation of apical membrane Na^+/H^+ exchange.

Inhibitors of tyrosine kinase pathways block stimulation of HCO_3^- absorption by hyposmolality. The signaling pathways involved in hyposmotic regulation of Na^+/H^+ exchange are not understood. Based on our previous demonstration that inhibition of apical Na^+/H^+ exchange activity and HCO_3^- absorption by hyperosmolality was blocked by tyrosine kinase inhibitors, we tested whether tyrosine kinase pathways also are involved in the stimulation by hyposmolality. Tubules were bathed with genistein or herbimycin A, inhibitors that selectively block tyrosine kinase-dependent regulation of HCO_3^- absorption in the MTAL (13, 39). The results in Figure 7 show that in the presence of 7 μM genistein or 1 μM herbimycin A, hyposmolality had no effect on HCO_3^- absorption (13.1 ± 0.7 pmol/min per millimeter inhibitor, vs. 13.4 ± 0.6 pmol/min per millimeter inhibitor plus hyposmolality, $n = 9$; $P = \text{NS}$). These results suggest that tyrosine kinase pathways play a role in the hyposmotic stimulation of HCO_3^- absorption.

To examine further whether tyrosine kinase pathways are involved in the hyposmotic regulation of HCO_3^- absorption, we tested the effect of molybdate, a potent tyrosine phosphatase inhibitor that blocks inhibition of HCO_3^- absorption by hyperosmolality (13, 40). In MTAL bathed with 50 μM sodium molybdate, removal of 50 mM mannitol from the lumen and bath had no effect on HCO_3^- absorption (10.0 ± 0.4 pmol/min per millimeter molybdate vs. 10.1 ± 0.5 pmol/min per millimeter molybdate plus hyposmolality, $n = 4$; $P = \text{NS}$). Taken together, these findings support a role for tyrosine phosphorylation in the stimulation of HCO_3^- absorption by hyposmolality.

Discussion

The regulation of epithelial Na^+/H^+ exchangers and their related functions by hyposmolality is poorly understood. The present study demonstrates that hyposmolality increases transepithelial HCO_3^- absorption in the MTAL through stimulation of apical membrane Na^+/H^+ exchange activity, attributable to stimulation of NHE3. Analysis of the mechanism of this stimulation showed that it is due to an increase in V_{\max} of the exchanger. The hyposmolality-induced increase in HCO_3^- absorption is mediated via a tyrosine kinase-dependent signaling pathway. These studies, to our knowledge, are the first to demonstrate stimulation of an epithelial Na^+/H^+ exchanger by hyposmolality and to provide evidence that the NHE3 isoform is regulated by hyposmotic stress. Moreover, our findings identify a previously unrecognized mechanism of Na^+/H^+ exchange regulation that may contribute importantly to changes in renal net acid and sodium excretion in a variety of pathophysiological conditions in which decreases in osmolality are manifest.

Hyposmolality increases HCO_3^- absorption through stimulation of apical membrane Na^+/H^+ exchange. The critical role of apical membrane Na^+/H^+ exchange in mediating hyposmotic stimulation of HCO_3^- absorption was demonstrated by 2 lines of evidence: (a) the stimulation was eliminated by lumen EIPA (Figure 4a); and (b) hyposmolality increased apical Na^+/H^+ exchange activity when assessed directly by measurement of lumen Na^+ -

dependent acid extrusion rates (Figure 5). The stimulation of the exchanger was due to an increase in V_{\max} . These findings indicate that hyposmotic stress triggers signaling pathways that are coupled directly to stimulation of the apical Na^+/H^+ exchanger. In principle, hyposmotic swelling could increase apical Na^+/H^+ exchange activity indirectly by decreasing pH_i or cell $[\text{Na}^+]$. We found, however, that hyposmolality stimulates the apical exchanger independent of pH_i (Figure 5) and in cells depleted of Na^+ , thereby precluding any differences in cell Na^+ activity (12). Thus, hyposmolality stimulates apical Na^+/H^+ exchange activity in the absence of a change in the driving force for the exchanger. These findings support the conclusion that hyposmolality increases V_{\max} of the exchanger by increasing the turnover rate or the transporter number through an intracellular signal transduction mechanism. As discussed below, this signaling mechanism may be tyrosine kinase dependent.

In the MTAL, transepithelial absorption of NaHCO_3 and NaCl are mediated through different apical membrane transporters: Na^+/H^+ exchange mediates NaHCO_3 absorption, whereas $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransport mediates NaCl absorption (18, 24, 33). Although these absorptive processes occur independently (18, 27), changes in one transport system could, in principle, influence the activity of the other system, such as through effects on cell Na^+ activity. In the present study, hyposmolality stimulated HCO_3^- absorption to a similar extent in the absence and presence of lumen furosemide, indicating that the stimulation occurs independent of $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransport-mediated NaCl absorption. This finding is consistent with our demonstration that hyposmolality stimulates HCO_3^-

absorption through a direct action on the kinetics of the apical Na^+/H^+ exchanger and provides further evidence that NaHCO_3 and NaCl absorption are regulated independently in the MTAL. We also found that the stimulation of HCO_3^- absorption was dependent on a decrease in the osmolality of the bath solution. This finding is consistent with previous studies demonstrating that osmotic-induced changes in cell volume and ion transport in the MTAL are transduced across the basolateral membrane but not across the apical membrane, because of the negligibly low apical membrane H_2O permeability (27, 32, 34, 35, 41). Our results comparing the effects of mannitol removal and NaCl removal indicate that the hyposmotic stimulation of HCO_3^- absorption is not mediated by a decrease in the solution ionic strength. Also, we have found that pretreatment with phosphatidylinositol-3 kinase inhibitors blocks the hyposmolality-induced stimulation of HCO_3^- absorption but does not prevent the increase in cell volume (D. Good, unpublished observations). This indicates that the increase in cell volume alone is not sufficient to cause stimulation of HCO_3^- absorption but does not address whether the increase in volume is necessary for the HCO_3^- transport stimulation. Recent studies in leukocytes indicate that hypertonic stimulation of NHE1 is dependent on alterations in cell size (42). Based on the above findings, we believe it is likely that increased cell volume is the signal leading to stimulation of HCO_3^- absorption in the MTAL; however, further work is needed to establish that the increases in cell volume and apical Na^+/H^+ exchange activity induced by hyposmolality are causally related.

Several observations indicate that the apical membrane Na^+/H^+ exchange activity responsible for HCO_3^- absorption in the MTAL is mediated by NHE3: (a) immunocytochemical studies using isoform-specific antibodies have localized NHE3 to the apical membrane of MTAL cells (16, 20); (b) the apical Na^+/H^+ exchange activity is relatively amiloride resistant and inhibited by hyperosmolality (12, 29), 2 distinguishing features of NHE3 (2, 4, 10, 14); and (c) chronic metabolic acidosis, which increases apical Na^+/H^+ exchange activity and HCO_3^- absorption in the MTAL (18), increases NHE3 protein expression (25). In the present study we demonstrate further that hyposmolality increased specifically the activity of an amiloride-resistant, HOE 694-insensitive apical membrane Na^+/H^+ exchanger, consistent with stimulation of NHE3. Recent studies suggest that the apical membrane of the MTAL also may express the amiloride-sensitive isoform NHE2 (36, 37). However, lumen addition of amiloride or HOE 694 at concentrations that should inhibit NHE2 virtually completely (2, 3) had no effect on HCO_3^- absorption under isosmotic (29) or hyposmotic (Figure 4b) conditions. Also, NHE2 was inhibited by hyposmolality in other systems (10, 23). Taken together, these findings strongly support the conclusion that hyposmolality increases HCO_3^- absorption in the MTAL through stimulation of NHE3.

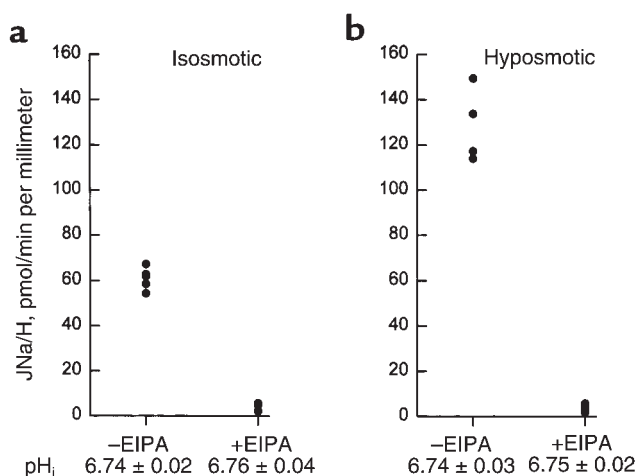


Figure 6 EIPA inhibits apical membrane Na^+/H^+ exchange activity. MTAL were studied in isosmotic (a) or hyposmotic (50 mM mannitol removed) (b) solutions. Apical Na^+/H^+ exchange rates ($J_{\text{Na}/\text{H}}$) were determined from rates of pH_i recovery after lumen Na^+ addition in the absence and presence of 50 μM lumen EIPA. Data points are values for single tubules. Data without EIPA are from Figure 5; $n = 4$ for each condition. Mean values are presented in Results. Values below figure panels are $\text{pH}_i \pm \text{SE}$ at which $J_{\text{Na}/\text{H}}$ was determined.

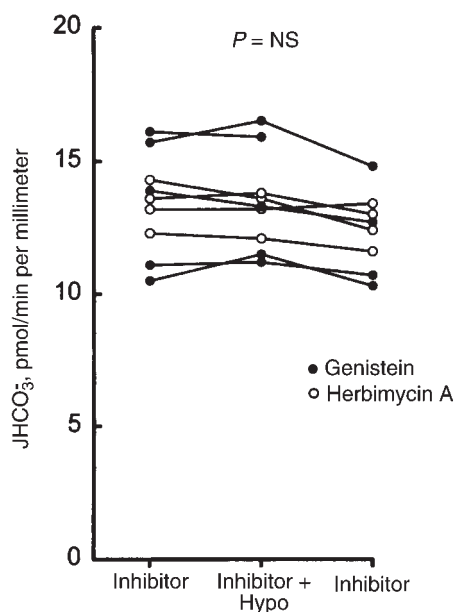


Figure 7
Tyrosine kinase inhibitors block stimulation of HCO_3^- absorption by hyposmolality. Hyposmolality was produced by removal of 50 mM mannitol from lumen and bath solutions. Genistein (7 μM , filled circles) or herbimycin A (1 μM , open circles) was present in the bath throughout the experiments. JHCO_3^- , data points, lines, and P value are as in Figure 1. Mean values are presented in Results.

In previous studies hyposmolality had no effect on NHE3 activity in NHE-transfected Chinese hamster ovary cells (10) or in LLC-PK₁ cells, a renal epithelial cell line (23). Thus, to our knowledge, our studies provide the first evidence that the NHE3 isoform is regulated by hyposmotic stress. Furthermore, they establish the physiological relevance of this regulation by demonstrating that it results in the stimulation of transepithelial NaHCO_3 absorption, a primary function of NHE3 in renal epithelia. At present, it is unclear whether the stimulation we observed represents a unique regulatory property of NHE3 in the MTAL. It is apparent, however, that the regulatory mechanisms that couple hyposmolality to the stimulation of NHE3 in the MTAL are absent or functionally inactive in the cell lines studied previously.

NHE isoforms are regulated differentially by hyperosmotic stress. In particular, hyperosmolality stimulates NHE1 and NHE2 but inhibits NHE3 (4, 7, 10–15). (The response of NHE2 to hyperosmolality is controversial, with some studies reporting stimulation [10, 11] and others inhibition [15].) In the present study we found that hyposmolality stimulates NHE3, an effect opposite to the inhibition of NHE1 and NHE2 by hyposmolality in other systems (10, 22, 23). Thus, although a degree of caution is warranted because the NHE activities were studied in different cell types, our results suggest that the differential regulation of NHEs observed for hyperosmotic stress also occurs in response to hyposmotic stress. It should be noted, however, that in a recent study in jejunal villus epithelial

cells, a biphasic response of NHE1 to hyposmotic stress was observed: a 5–7% hyposmotic dilution increased exchanger activity, whereas a 30% hyposmotic dilution decreased exchanger activity (22). It is presently unclear whether this dilution dependence is applicable to other cell types or to other NHE isoforms. The decreases in osmolality used in the present study represent a reasonable estimate of the decreases in plasma osmolality that typically are observed in clinically relevant hyposmolar states (see below).

The signal transduction pathways that mediate hyposmotic regulation of Na^+/H^+ exchange are unknown. The results of the present study indicate that tyrosine kinase pathways are involved in hyposmotic stimulation of HCO_3^- absorption in the MTAL. Several observations support this view: (a) the stimulation is blocked completely by genistein and herbimycin A, 2 chemically unrelated tyrosine kinase inhibitors with different mechanisms of action (13, 43, 44); (b) these inhibitors block hyposmotic stimulation at low concentrations (< 10 μM) that selectively inhibit tyrosine kinases (13, 43, 44). The specificity of genistein and herbimycin A has been established previously in the MTAL by the observations that these agents selectively block tyrosine kinase-dependent HCO_3^- transport regulation, with no effects on regulation through cAMP- or protein kinase C-dependent pathways (13, 39); (c) hyposmotic stimulation was blocked by the tyrosine phosphatase inhibitor molybdate (40), providing further support for a role for tyrosine phosphorylation. In previous studies we demonstrated that the effect of hyperosmolality to inhibit HCO_3^- absorption through inhibition of apical Na^+/H^+ exchange also is blocked by tyrosine kinase inhibitors (13), suggesting that tyrosine phosphorylation plays a generalized role in the osmotic regulation of HCO_3^- absorption in the MTAL. In addition, other physiologic effects of hyposmolality have been reported to depend on tyrosine phosphorylation, including activation of Cl^- channels in intestinal and endothelial cells (45, 46) and alkalization of endocytic vesicles in hepatocytes (47). In the MTAL tyrosine kinase inhibitors increase, whereas tyrosine phosphatase inhibitors decrease, basal HCO_3^- absorption (13). Based on these findings, we speculate that the stimulation of HCO_3^- absorption by hyposmolality may involve a decrease in tyrosine phosphorylation as a critical step in the regulatory pathway. For example, hyposmolality could reduce tyrosine phosphorylation through stimulation of tyrosine phosphatase activity, a mechanism that could explain the ability of both tyrosine kinase and tyrosine phosphatase inhibitors to block hyposmotic stimulation of HCO_3^- absorption. Based on our findings that hyposmolality increases HCO_3^- absorption through stimulation of NHE3 and that tyrosine kinase inhibitors block completely the hyposmotic stimulation of HCO_3^- absorption, it is reasonable to postulate that hyposmotic stress may be coupled directly to the activation of NHE3 via a tyrosine kinase pathway. However, our results do not rule

out the alternative possibility that hyposmolality may act via a tyrosine kinase pathway to modify the activity of another transport pathway, such as basolateral HCO_3^- efflux, that may contribute to the stimulation of HCO_3^- absorption. Important goals for future studies will be to identify proteins that exhibit changes in tyrosine phosphorylation in response to hyposmotic stress in the MTAL and to determine whether hypo- and hyperosmolality regulate HCO_3^- absorption through similar or distinct tyrosine kinase pathways. The precise role of tyrosine kinase pathways in the osmotic regulation of apical Na^+/H^+ exchange (NHE3) activity also remains to be determined.

Although hypo- and hyperosmolality both influence MTAL HCO_3^- absorption via tyrosine kinase pathways, additional evidence indicates that these stimuli regulate apical Na^+/H^+ exchange activity through distinct mechanisms. Hyposmolality stimulates exchanger activity by increasing V_{max} with no effect on the apparent affinity for intracellular H^+ (Figure 5). In contrast, hyperosmolality inhibits exchanger activity predominantly by decreasing the apparent affinity for internal H^+ , reflected by an acid-direction shift in the pH_i -dependence curve (12). These different kinetic mechanisms suggest that the contrary effects of hypo- and hyperosmolality on apical Na^+/H^+ exchange activity are not simply the result of opposite changes in a common regulatory pathway. This conclusion is supported further by our recent demonstration that inhibitors of phosphatidylinositol 3-kinase blocked completely the stimulation of HCO_3^- absorption by hyposmolality but had no effect on the inhibition of HCO_3^- absorption by hyperosmolality (48). It appears, therefore, that in the MTAL, hyposmolality and hyperosmolality regulate NHE3 activity through distinct signal transduction pathways that modify exchanger activity through different kinetic mechanisms.

Physiological implications and clinical relevance. Our finding that hyposmolality directly stimulates apical membrane Na^+/H^+ exchange (NHE3) activity in the MTAL identifies a previously undescribed mechanism that may contribute importantly to changes in renal electrolyte excretion in a variety of physiological and pathophysiological conditions. Cells in the renal medulla are routinely exposed to rapid and pronounced changes in osmolality because of the function of the urinary concentrating mechanism that controls H_2O excretion (26). The osmolality of plasma or interstitial fluid in the outer medulla has not been measured under any conditions; thus, the extent to which peritubular osmolality falls in this region in hyposmolal states is not known. Our results demonstrate, however, that HCO_3^- absorption in the MTAL is stimulated by a small decrease in osmolality (25 mosmol/kg H_2O) that is well within the range of values observed clinically in serum in hyponatremic disorders (49). In addition, the volume of renal medullary cells normally is adapted to hyperosmolality in vivo; thus, a decrease in medullary osmolality in vivo would constitute a cell stress similar to that achieved with

hyposmolality in the current study (41, 50). Furthermore, we have shown that stimulation of HCO_3^- absorption in the MTAL occurs in response to a decrease in the concentration of NaCl , the solute primarily responsible for changes in the osmolality of the renal outer medullary interstitium in vivo (26). It is likely, therefore, that the transport effects and signaling pathways induced by hyposmolality in the present study are relevant to the response of the MTAL to physiologic decreases in extracellular osmolality in vivo, irrespective of whether those decreases achieve hyposmotic levels (26, 41, 50). Stimulation of apical membrane Na^+/H^+ exchange activity and HCO_3^- absorption in the MTAL by a fall in osmolality could contribute to the urine acidifying effects of loop-acting diuretics (51, 52) and to the increase in urinary net acid excretion that maintains constant the plasma HCO_3^- concentration during hypotonic volume expansion (53). Our findings have additional implications for regulation of sodium balance if hyposmolality stimulates apical Na^+/H^+ exchange activity in other nephron segments such as the proximal tubule, where NHE3 mediates NaCl absorption as well as NaHCO_3 absorption (17, 19). For example, hyposmolality at a level similar to that used in the present study is a common side effect of treatment with diuretic drugs (49, 52). An effect of hyposmolality to stimulate directly apical Na^+/H^+ exchange activity and NaCl absorption in the proximal tubule could contribute to the development and maintenance of diuretic resistance. Hyposmolality also is a common feature of chronic edematous states such as congestive heart failure and cirrhosis (49, 52). Hyposmotic stimulation of apical Na^+/H^+ exchange could thus contribute to the maladaptive renal sodium retention observed in these conditions.

Acknowledgments

We are grateful to T. Wang and G. Giebisch for providing the HOE 694. We also thank L. Reuss for critical reading of the manuscript. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-38217.

1. Grinstein, S., Rotin, D., and Mason, M.J. 1989. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochim. Biophys. Acta.* **988**:73-97.
2. Noel, J., and Pouyssegur, J. 1995. Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. *Am. J. Physiol.* **268**:C283-C296.
3. Yun, C.H.C., et al. 1995. Mammalian Na^+/H^+ exchanger gene family: structure and function studies. *Am. J. Physiol.* **269**:G1-G11.
4. Wakabayashi, S., Shigekawa, M., and Pouyssegur, J. 1997. Molecular physiology of vertebrate Na^+/H^+ exchangers. *Physiol. Rev.* **77**:51-74.
5. Orłowski, J., and Grinstein, S. 1997. Na^+/H^+ exchangers of mammalian cells. *J. Biol. Chem.* **272**:22373-22376.
6. Hoffman, E.K., and Simonsen, L.O. 1989. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69**:315-382.
7. Grinstein, S., Woodside, M., Sardet, C., Pouyssegur, J., and Rotin, D. 1992. Activation of Na^+/H^+ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. *J. Biol. Chem.* **267**:23823-23828.
8. Biemesderfer, D., Reilly, R.F., Exner, M., Igarashi, P., and Aronson, P.S. 1992. Immunocytochemical characterization of Na^+/H^+ exchanger isoform NHE-1 in rabbit kidney. *Am. J. Physiol.* **263**:F833-F840.
9. Bookstein, C., et al. 1994. A unique sodium-hydrogen exchange isoform (NHE4) of the inner medulla of the rat kidney is induced by hyperosmolality. *J. Biol. Chem.* **269**:29704-29709.

10. Kapus, A., Grinstein, S., Wasan, S., Kandasamy, R., and Orlowski, J. 1994. Functional characterization of three isoforms of the Na⁺/H⁺ exchanger stably expressed in Chinese hamster ovary cells. *J. Biol. Chem.* **269**:23544–23552.
11. Soleimani, M., Singh, G., Bizal, G.L., Gullans, S.R., and McAteer, J.A. 1994. Na⁺/H⁺ exchanger isoforms NHE-2 and NHE-1 in inner medullary collecting duct cells. Expression, functional localization, and differential regulation. *J. Biol. Chem.* **269**:27973–27978.
12. Watts, B.A., III, and Good, D.W. 1994. Apical membrane Na⁺/H⁺ exchange in rat medullary thick ascending limb: pH_i-dependence and inhibition by hyperosmolality. *J. Biol. Chem.* **269**:20250–20255.
13. Good, D.W. 1995. Hyperosmolality inhibits HCO₃⁻ absorption in rat medullary thick ascending limb via a protein-tyrosine kinase-dependent pathway. *J. Biol. Chem.* **270**:9883–9889.
14. Soleimani, M., et al. 1994. Effect of high osmolality on Na⁺/H⁺ exchange in renal proximal tubule cells. *J. Biol. Chem.* **269**:15613–15618.
15. Nath, S.K., et al. 1996. Hyperosmolality inhibits the Na⁺/H⁺ exchanger isoforms NHE2 and NHE3: an effect opposite to that on NHE1. *Am. J. Physiol.* **270**:G431–G441.
16. Amemiya, M., et al. 1995. Expression of NHE-3 in the apical membrane of rat renal proximal tubule and thick ascending limb. *Kidney Int.* **48**:1206–1215.
17. Alpern, R.J., Moe, O.W., and Preisig, P.A. 1995. Chronic regulation of the proximal tubule Na/H antiporter: from HCO₃⁻ to SRC. *Kidney Int.* **48**:1386–1396.
18. Good, D.W., and Watts, B.A., III. 1996. Functional roles of apical membrane Na⁺/H⁺ exchange in rat medullary thick ascending limb. *Am. J. Physiol.* **270**:F691–F699.
19. Wu, M.-S., Biemesderfer, D., Giebisch, G., and Aronson, P.S. 1996. Role of NHE3 in mediating renal brush border Na⁺/H⁺ exchange: adaptation to metabolic acidosis. *J. Biol. Chem.* **271**:32749–32752.
20. Biemesderfer, D., et al. 1997. Monoclonal antibodies for high-resolution localization of NHE3 in adult and neonatal rat kidney. *Am. J. Physiol.* **273**:F289–F299.
21. Green, J., Yamaguchi, D.T., Kleeman, C.R., and Muallem, S. 1988. Selective modification of the kinetic properties of Na⁺/H⁺ exchanger by cell shrinkage and swelling. *J. Biol. Chem.* **263**:5012–5015.
22. MacLeod, R.J., and Hamilton, J.R. 1996. Activation of Na⁺/H⁺ exchange is required for regulatory volume decrease after modest “physiological” volume increases in jejunal villus epithelial cells. *J. Biol. Chem.* **271**:23138–23145.
23. Amlal, H., Goel, A., and Soleimani, M. 1998. Activation of H⁺-ATPase by hypotonicity: a novel regulatory mechanism for H⁺ secretion in IMCD cells. *Am. J. Physiol.* **275**:F487–F501.
24. Good, D.W. 1993. The thick ascending limb as a site of renal bicarbonate reabsorption. *Semin. Nephrol.* **13**:225–235.
25. Laghmani, K., et al. 1997. Chronic metabolic acidosis enhances NHE-3 protein abundance and transport activity in rat thick ascending limb by increasing NHE-3 mRNA. *J. Clin. Invest.* **99**:24–30.
26. Knepper, M.A., and Rector, F.C., Jr. 1996. Urine concentration and dilution. In *The kidney*. 5th edition. Saunders, Philadelphia, PA. 532–570.
27. Good, D.W. 1992. Effects of osmolality on bicarbonate absorption by medullary thick ascending limb of the rat. *J. Clin. Invest.* **89**:184–190.
28. Watts, B.A., III, and Good, D.W. 1994. Effects of ammonium on intracellular pH in rat medullary thick ascending limb: mechanisms of apical membrane NH₄⁺ transport. *J. Gen. Physiol.* **103**:917–936.
29. Good, D.W., George, T., and Watts, B.A., III. 1995. Basolateral membrane Na⁺/H⁺ exchange enhances HCO₃⁻ absorption in rat medullary thick ascending limb: Evidence for functional coupling between basolateral and apical membrane Na⁺/H⁺ exchangers. *Proc. Natl. Acad. Sci. USA.* **92**:12525–12529.
30. Krapf, R. 1988. Basolateral membrane H/OH/HCO₃ transport in the rat cortical thick ascending limb. Evidence for an electrogenic Na/HCO₃ cotransporter in parallel with a Na/H antiporter. *J. Clin. Invest.* **82**:234–241.
31. Boyarsky, G., Ganz, M.B., Cragoe, E.J., Jr., and Boron, W.F. 1990. Intracellular-pH dependence of Na⁺/H⁺ exchange and acid loading in quiescent and arginine vasopressin-activated mesangial cells. *Proc. Natl. Acad. Sci. USA.* **87**:5921–5924.
32. Hebert, S.C. 1986. Hypertonic cell volume regulation in mouse thick limbs. I. ADH dependency and nephron heterogeneity. *Am. J. Physiol.* **250**:C907–C919.
33. Greger, R. 1985. Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. *Physiol. Rev.* **3**:760–796.
34. Hebert, S.C., Culpepper, R.M., and Andreoli, T.E. 1981. NaCl transport in mouse medullary thick ascending limbs. III. Modulation of the ADH effect by peritubular osmolality. *Am. J. Physiol.* **241**:F443–F451.
35. Sun, A.M., Saltzberg, S.N., Kikeri, D., and Hebert, S.C. 1990. Mechanisms of cell volume regulation by the mouse medullary thick ascending limb of Henle. *Kidney Int.* **38**:1019–1029.
36. Sun, A.M., et al. 1997. Na⁺/H⁺ exchanger isoform 2 (NHE2) is expressed in the apical membrane of the medullary thick ascending limb. *J. Membr. Biol.* **160**:85–90.
37. Chambrey, R., et al. 1998. Immunolocalization of the Na⁺/H⁺ exchanger isoform NHE2 in rat kidney. *Am. J. Physiol.* **275**:F379–F386.
38. Watts, B.A., III, George, T., and Good, D.W. 1999. Nerve growth factor inhibits HCO₃⁻ absorption in renal thick ascending limb through inhibition of basolateral membrane Na⁺/H⁺ exchange. *J. Biol. Chem.* **274**:7841–7847.
39. Good, D.W. 1998. Nerve growth factor regulates HCO₃⁻ absorption in thick ascending limb: modifying effects of vasopressin. *Am. J. Physiol.* **274**:C931–C939.
40. Walton, K.M., and Dixon, J.E. 1993. Protein tyrosine phosphatases. *Annu. Rev. Biochem.* **62**:101–120.
41. Hebert, S.C., and Sun, A. 1988. Hypotonic cell volume regulation in mouse medullary thick ascending limb: effects of ADH. *Am. J. Physiol.* **255**:F962–F969.
42. Krump, E., Nikitas, K., and Grinstein, S. 1997. Induction of tyrosine phosphorylation and Na⁺/H⁺ exchanger activation during shrinkage of human neutrophils. *J. Biol. Chem.* **272**:17303–17311.
43. Akiyama, T., and Ogawara, H. 1991. Use and specificity of genistein as an inhibitor of protein tyrosine kinases. *Methods Enzymol.* **201**:362–370.
44. Uehara, Y., and Fukazawa, H. 1991. Use and selectivity of herbimycin A as inhibitor of protein-tyrosine kinases. *Methods Enzymol.* **201**:370–379.
45. Tilly, B.C., et al. 1996. Activation of the osmo-sensitive chloride conductance involves p21^{tho} and is accompanied by a transient reorganization of the F-actin cytoskeleton. *Mol. Biol. Cell.* **7**:1419–1427.
46. Voets, T., et al. 1998. Regulation of a swelling-activated chloride current in bovine endothelium by protein tyrosine phosphorylation and G proteins. *J. Physiol. (Lond)*. **506**:341–352.
47. Schreiber, R., and Häussinger, D. 1995. Characterization of the swelling-induced alkalization of endocytic vesicles in fluorescein isothiocyanate-dextran-loaded rat hepatocytes. *Biochem. J.* **309**:19–24.
48. Good, D.W., and Watts, B.A., III. 1998. Hyposmolality stimulates apical membrane Na⁺/H⁺ exchange activity via a phosphatidylinositol 3-kinase-dependent pathway in rat medullary thick ascending limb. *J. Am. Soc. Nephrol.* **9**:6A. (Abstr.)
49. Kumar, S., and Berl, T. 1998. Sodium. *Lancet.* **352**:220–228.
50. Burg, M.B., Kwon, E.D., and Kültz, D. 1997. Regulation of gene expression by hypertonicity. *Annu. Rev. Physiol.* **59**:437–455.
51. DuBose, T.D., Jr., and Good, D.W. 1988. Effects of diuretics on renal acid-base transport. *Semin. Nephrol.* **8**:282–294.
52. Wilcox, C.S. 1996. Diuretics. In *The kidney*. 5th edition. Saunders, Philadelphia, PA. 2299–2330.
53. Lowance, D.C., Garfinkel, H.B., and Mattern, W.D. 1972. The effect of chronic hypotonic volume expansion on the renal regulation of acid-base equilibrium. *J. Clin. Invest.* **51**:2928–2940.