Gastrointestinal Distribution and Kinetic Characterization of the Sodium-Hydrogen Exchanger Isoform 8 (NHE8)

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Key Words

NHE8 kinetics • PS120 cells • GI distribution

Abstract

NHE8 is a newly identified NHE isoform expressed in rat intestine. To date, the kinetic characteristics and the intestinal segmental distribution of this NHE isoform have not been studied. This current work was performed to determine the gene expression pattern of the NHE8 transporter along the gastrointestinal tract, as well as its affinity for Na⁺, H⁺, and sensitivity to known NHE inhibitors HOE694 and S3226. NHE8 was differentially expressed along the GI tract. Higher NHE8 expression was seen in stomach, duodenum, and ascending colon in human, while higher NHE8 expression was seen in jejunum, ileum and colon in adult mouse. Moreover, the expression level of NHE8 is much higher in the stomach and jejunum in young mice compared with adult mice. To evaluate the functional characteristics of NHE8, the pH indicator SNARF-4 was used to monitor the rate of intra-cellular pH (pH_i) recovery after an NH₄Cl induced acid load in NHE8 cDNA transfected PS120 cells. The NHE8 cDNA transfected cells exhibited a sodium-dependent proton exchanger activity having a Km for pH_i

of ~ pH 6.5, and a Km for sodium of ~ 23mM. Low concentration of HOE694 (1 μM) had no effect on NHE8 activity, while high concentration (10 μM) significantly reduced NHE8 activity. In the presence of 80 μM S3226, the NHE8 activity was also inhibited significantly. In conclusion, our work suggests that NHE8 is expressed along the gastrointestinal tract and NHE8 is a functional Na⁺/H⁺ exchanger with kinetic characteristics that differ from other apically expressed NHE isoforms.

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Introduction

Sodium-hydrogen exchangers (NHEs) are integral transmembrane proteins that exchange extracellular Na⁺ for intracellular H⁺ and are present in all mammalian cells. NHEs have multiple cellular functions including intracellular pH homeostasis, cell volume regulation, and electroneutral NaCl absorption in epithelia. There are nine NHE isoforms (NHE1-9) identified from mammalian cells [1-14]. These NHEs have different tissue distributions, membrane localization, inhibitor sensitivities, and physiological regulation [15]. Additionally, these NHEs play roles in several disease states, including diarrheal disorders, hypertension, and cardiac ischemia [16]. The

investigation of NHEs is of clinical relevance due to the potential relationship in their expression, activity and the development of these disorders in humans.

To date, five NHEs have been identified in intestine. NHE1 is the most ubiquitous expressed NHE isoform, with its mRNA expressed in nearly all mammalian cells [16]. NHE1 protein is localized to the basolateral membrane in intestinal epithelial cells [17, 18] and also in the kidney epithelium [19]. NHE1 regulates cell volume and intracellular pH (pH_i) [13]. NHE2 mRNA is detected in multiple organs including kidney, liver, intestine, brain, and testis [20]. NHE2 protein is localized to the apical, or brush-border membrane (BBM), in intestinal epithelia and in renal cortical epithelium [21]. By mediating net Na⁺ transport across the apical membrane, NHE2 plays a significant role in intestinal Na⁺ absorption and cell volume regulation in renal medullary collecting duct cells [21]. NHE3 protein is detected at the intestinal BBM in rabbit, human and rat [15], and NHE3 mRNA is expressed in the kidney and the intestine [22, 23]. Consequently, NHE3 has been shown to be important for transepithelial Na⁺ absorption in the mammalian gastrointestinal tract and kidney. NHE4 message has been shown to be expressed in rat stomach, intestine, kidney, uterus, and brain. Moreover, one study suggests that NHE4 protein is expressed on the basolateral membrane in renal inner medullary collecting ducts [24]. However the exact function and localization of this isoform remain unknown. Another member of the NHE family, NHE8, was cloned recently from rat intestine [14] and mouse kidney [7]. The NHE8 isoform was localized on the apical membrane of intestinal and renal epithelial cells [14, 25]. However, it is not clear if this transporter mediates sodium dependent proton exchange. Thus, the current study was designed to determine the intestinal segmental distribution of NHE8 and to characterize the NHE8 transport kinetics.

In this communication, we demonstrate that NHE8 is expressed differentially along the gastrointestinal tract, and that NHE8 protein mediates Na⁺-dependent H⁺ exchange in PS120 cells. We further show that NHE8 exchanger exhibits different transport kinetics as compared to NHE2 and NHE3.

Materials and Methods

Northern Blot Analyses

Mouse RNA blots were purchased from Zyagen laboratories (San Diego, CA) and human RNA blots were purchased from BD Biosciences Clontech (Palo Alto, CA). According to the company information, multiple subjects were

used for RNA purification, and equal amount RNA was loaded in each line. These blots were utilized for Northern blot analyses. The rat and human NHE8 cDNA were radiolabeled as the probe for NHE8 mRNA detection. Northern blot analyses were performed under high stringency washing conditions as described previously [14]. Blots were exposed to a phosphoimaging screen and band intensities were determined with Quantity One Software (FX Molecular Imager; Biorad; Hercules, CA).

Plasmid Construction

The full length rat intestinal NHE8 cDNA was subcloned into Mammalian Lumio Gateway Vectors (Invitrogen) following the manufacturer's protocol. Briefly, rat NHE8 primers were designed and rat NHE8 cDNA was PCR amplified and cloned into pENTR/D-TOPO vector. The cloned PCR product was sequenced in both directions to ensure accurate duplication. The NHE8 cDNA was then cloned into gateway destination vectors pcDNA6.2/nLumio-DEST with Lumio tag at the N-terminus of NHE8 cDNA (pcDNA6.2/nLumio-NHE8) or into pcDNA6.2/cLumio-DEST with Lumio tag at the C-terminus of NHE8 cDNA (pcDNA 6.2/cLumio-NHE8).

Cell Culture

PS120 cells were a gift from Dr. Pouyssegur (University of Nice; Nice, France). They are NHE-deficient fibroblast-derived cells, originally isolated from the lung of the Chinese hamster [26]. Cells were cultured at 37°C in a 95% air-5% CO₂ atmosphere in DME medium supplemented with 1% Penicillin-Streptomycin and 10% fetal bovine serum. Cells were passaged every 48 hours. Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA).

Stable Transfection

Rat NHE8 cDNA construct was introduced into PS120 cells by the Effectene-mediated transfection method. Twenty-four hours after transfection, the cells were treated by acid load to select NHE8 expressing cells. Briefly, cells were incubated with acid medium (in mM: 50 NH₄Cl, 72 choline, 4.9 KCl, 1 CaCl₂, 2.5 MgCl₂, and 20 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES); pH 7.4) for 1 h at 37°C. Cells were rinsed twice with choline solution (in mM: 122 choline, 4.9 KCl, 1 CaCl₂, 2.5 Mg₂SO₄, and 20 HEPES; pH 7.4) and incubated in NaCl solution (in mM: 122 NaCl, 4.9 KCl, 1 CaCl₂, 2.5 Mg₂SO₄, and 20 HEPES; pH 7.4) for 1 h at 37°C. Cells were then rinsed twice with DMEM-10% FBS-1% PS and incubated at 37°C for 24 h. The acid selection protocol was repeated three times. Cells that survived acid selection were used for NHE8 functional characterization study.

NHE8 kinetics in transfected PS120 cells utilizing intracellular pH (pH_i) measurements

pH_i was assessed by monitoring the fluorescence emission of the pH-sensitive dye seminaphthorhodofluor-4 (SNARF-4, Molecular Probes, Eugene, OR) with the method published previously by our laboratory [27]. This probe was utilized for these measurements, because its pK_a for H⁺ (~6.4) is optimal for monitoring pH_i during the recovery phase after a

Fig. 1. Segmental Expression of NHE8 Gene along the gastrointestinal tract. Northern hybridization was performed on the human (Top) and mouse (Bottom) RNA blots with ^{32}P labeled NHE8 cDNA specific probes. Northern blot analysis indicated that NHE8 mRNA is expressed along the gastrointestinal tract both in human and mouse with different abundances. Human NHE8 mRNA was detected at 4 kb and 7 kb. Mouse NHE8 mRNA was detected at 4 kb.

H^+ load. Cells were grown on no. 1 glass coverslips and incubated with $5 \mu\text{M}$ SNARF-4-AM for 15 min at 37°C in a 5% CO_2 -equilibrated incubator. Cells were then washed once with HBSS (in mM: 5 KCl, 0.3 KH_2PO_4 , 138 NaCl, 0.2 NaHCO_3 , 0.3 Na_2HPO_4 , 10 HEPES, 1.3 CaCl_2 , 0.4 MgSO_4 , 5.6 glucose, and 5 glutamine, pH 7.4) and incubated in HBSS for 15 min at 37°C to allow for full cleavage of the dye. Coverslips containing SNARF-4-loaded cells were mounted in a temperature-regulated chamber and transferred to the microscope stage. An Olympus IX-70 microscope equipped with a x40, 1.4 NA objective was used to acquire light emitted from the cells. A Multispectral Imager (Optical Insights, Albuquerque, NM) was used to split the emission beam, forming side-by-side images of emitted light at 640 nm (basic pH wavelength) and 570 nm (acidic pH wavelength) that were acquired on a CCD imaging array (CoolSnap, Photometrics, Tucson, AZ). The ratio of fluorescence intensity (640 nm/570 nm) was measured for individual cells, and these ratios were subsequently converted to pH_i by means of an in situ-derived calibration curve [28]. The relationship between the SNARF-4 fluorescence ratio and pH_i was fitted by using the following equation: $\text{pH}_i = \text{p}K + \log[(R - R_{\min}) / (R_{\max} - R)]$, where R represents the experimentally measured ratio of 640 nm/570 nm, $R_{\min}$ is the ratio measured at the most acidic pH_i , and $R_{\max}$ is the ratio measured at the most basic pH_i .

Na^+ -dependent pH_i recovery was analyzed by measuring the rate of pH_i recovery in HBSS after an acid load in the presence or absence of Na^+ . As a standard protocol, SNARF-4 loaded cells were incubated in the presence of 40 mM NH_4Cl for 5 min, and then the medium was replaced with an NH_4Cl -free HBSS. On removal of external NH_4Cl , NH_3 diffuses from the cells, leaving an intracellular acid load. The pH_i recovery rate was assessed by monitoring the pH_i change in the first 2 min after NH_4Cl is replaced with $\text{NH}_4\text{Cl}/\text{HCO}_3^-$ -free HBSS. To determine the sodium dependence of NHE8, all sodium was replaced with 138 mM methylglucamine in the wash solution, then the pH_i recovery was initiated by adding back various concentrations of sodium. To determine the effect of cellular pH on NHE8 activity, pH_i recovery rates were analyzed for cells with different initial pH_i ranging from pH6.2 to pH7.2. To determine the sensitivity of NHE8 to known NHE inhibitors, the pH_i recovery measurement was performed in the presence of HOE694 (NHE1 and NHE2 inhibitor), or S3226 (NHE3 inhibitor). To determine the effect of phorbol ester on NHE8 activity, NHE8 expressing PS120 cells were treated with 100 nM PMA for 60 minutes at 37°C before measuring pH_i recovery from acid load.

Curve Fit and Statistical Analysis

All curves used for determining kinetic constants were fit by non-linear regression using SigmaPlot (Jandel Scientific). ANOVA Post Hoc Tests (StatView 5.0.1 version, SAS Institute Inc.; Cary, NC) were conducted to compare values of the experimental data. P values of < 0.05 were considered significant.

Results

NHE8 Gene Expression along the Gastrointestinal Tract

Northern hybridizations were performed on human and mouse RNA blots with ^{32}P labeled NHE8 cDNA specific probes. Northern blot analysis indicated that a higher NHE8 expression was detected in human stomach, duodenum, and ascending colon (Fig. 1). In mouse, a higher NHE8 expression was observed in jejunum, ileum and colon. The expression level of NHE8 is much higher in the stomach and jejunum in young mice compared with adult mice (Fig. 2).

Characterization of Rat NHE8 Expression in Transfected PS120 Cells

PS120 cells were transfected with a vector or rat NHE8 cDNA construct by an Effectene-mediated method. NHE8 cDNA transfected PS120 cells were able to withstand acid selection while the vector-only transfected cells died. To characterize NHE8 function in transfected PS120 cells, the rate of pH_i recovery after an acid load was analyzed. NHE8 expressing PS120 cells loaded with SNARF-4 were incubated with NH_4Cl for 5 minutes, then washed with Na^+ -free HBSS for 2 minutes

Fig. 2. Ontagenic Expression of NHE8 in mouse Intestine. Northern hybridization was performed on RNA blot loaded with different aged mice samples, with mouse NHE8 specific probes. Northern blot data indicated that higher NHE8 expression was detected in the intestine from young mice. Mouse NHE8 mRNA was detected at 4 kb.

Fig. 3. Characterization of NHE8 Activity in Transfected PS120 Cells. To characterize NHE8 function in transfected PS120 cells, the rate of recovery in pH_i after an acid load was investigated. PS120 cells transfected with rat NHE8 cDNA and loaded with SNARF-4 were incubated with 40mM NH_4Cl in HBSS for 5 minutes, and then washed with sodium-free HBSS for 2 minutes. The pH_i recovery was measured for the duration of 2 minutes after adding HCO_3^- -free HBSS containing Na^+ . The values shown are mean $\pm$ SEM from 20 cells.

Fig. 4. Sodium Dependence of the NHE8 Activity. Na^+/H^+ exchanger activity mediated by rat NHE8 expressed in PS120 cells was measured in the presence of varying concentrations of sodium. PS120 cells were loaded with 40mM NH_4Cl in HBSS for 5 minutes, and then washed with sodium-free HBSS for 2 minutes before switching to sodium-containing HBSS. The sodium concentrations tested in this experiment were 0, 2.8, 5.6, 14, 28, 56, and 140 mM. The rate of pH_i recovery was analyzed during the initial 30 second following addition of sodium-containing HBSS. The values for recovery rates are averages from 7-10 cells at each concentration, and are presented as mean $\pm$ SEM.

to induce an intracellular acid load without the possibility of recovery (NHE and Cl^-/HCO_3^- transport inhibited). These acid loaded cells were then incubated in Na^+ containing HCO_3^- -free HBSS to activate NHE mediated H^+ efflux. Fig. 3 shows a time course of sodium-dependent pH_i recovery mediated by NHE8 protein in PS120 cells. A linear recovery rate occurred in the initial 30 seconds which reached a plateau after 60 seconds bringing pH_i back to near resting values. There was no recovery after acid load in nontransfected or vector transfected PS120 cells (data not shown). This observation demonstrates that the expressed NHE8 protein has sodium-hydrogen exchanger activity. These data also demonstrate that the lumio-tagged NHE8 protein can be expressed in PS120 cells, and these transfected cells can be useful as an *in vitro* model to study NHE8 function.

Sodium Dependent Kinetics of the Rat NHE8 Exchanger

The effect of Na^+ ion on pH_i recovery in NHE8 cDNA expressing PS120 cells was explored by measuring the rate of pH_i recovery as a function of Na^+ concentration. In NHE8 expressing PS120 cells, the Na -dependent pH_i recovery was measured after activation of NHE dependent transport with varying concentrations of sodium. There was a clear relationship between the initial pH_i recovery rate and the sodium concentration. No pH_i recovery was observed after an acid load at low extracellular Na^+ concentrations (≤ 5.6 mM). However, a substantial pH_i recovery was detected at higher Na^+ concentrations (> 5.6 mM). The pH_i recovery rate reached a plateau when the Na^+ concentration in the HBSS was higher than 56 mM. The K_m for Na^+ of rat

Fig. 5. Effect of Intracellular pH (pH_i) on the NHE8 Activity. PS120 cells transfected with rat NHE8 were loaded with 40mM NH₄Cl in HBSS for 5 minutes, and then washed with sodium-free HBSS for 2 minutes before adding sodium-containing HBSS. The rate of pH_i recovery was analyzed during the initial 30 second following addition of sodium-containing HBSS. The pH_i recovery rate was plotted against the intracellular pH measured immediately prior to the activation of recovery. The values shown are mean ± SEM from 5-7 cells at each pH point.

NHE8 protein in PS120 cells was estimated at 23mM (Fig. 4).

Proton Kinetics of the rat NHE8 exchanger

The effect of cellular pH on NHE8 exchanger activity was also tested in NHE8 expressing PS120 cells. The rate of pH_i recovery was analyzed in cells incubated in Na⁺-containing HBSS buffer, but at different acid loads, with initial pH_i ranging from pH6.2 to pH7.3. The sodium-dependent pH_i recovery rate was significantly affected by intracellular pH. Maximal activation of NHE8 was observed in cells with initial pH_i lower than pH6.5, while inactivation of NHE8 was seen when the cellular pH was above pH7.2. The K_m for pH_i of rat NHE8 protein in PS120 cells was estimated to be pH6.5 (Fig. 5).

Effect of HOE694 on the Activity of NHE8 in PS120 Cells

HOE 694 is a benzyl guanidine derivative that specifically inhibits NHE1 and NHE2. In PS120 cells, NHE1 activity is inhibited by HOE694 with K_i of 0.16 μM, while the NHE2 activity is inhibited by HOE694 with K_i of 5 μM [29]. In our experiments, we tested the sensitivity of NHE8 in PS120 cells by exposing these cells to varying concentrations of HOE694 during the NH₄Cl washout phase and the pH_i recovery phase. As shown in Fig. 6, 1 μM HOE 694 had no significant

Fig. 6. HOE694 Inhibition on the NHE8 Activity. PS120 cells transfected with NHE8 were loaded with 40mM NH₄Cl in HBSS for 5 minutes, and then washed with sodium-free HBSS for 2 minutes before adding sodium-containing HBSS. The rate of pH_i recovery was analyzed during the initial 30 second following addition of sodium-containing HBSS in the presence or absence of HOE694. HOE694 was included both during the NH₄Cl washout phase and the recovery phase. The relative changes on pH_i recovery at 30 second were calculated by dividing ΔpH_i from HOE694 treatment groups by control group. Data were collected from 10-15 cells at each concentration. *p < 0.02 for 10 μM HOE694 v.s. 0 μM HOE694 and 1 μM HOE694.

effect on NHE8 activity, while 10 μM HOE694 abolished NHE8 activity in PS120 cells.

Effect of S3226 on the Activity of NHE8 in PS120 Cells

S3226 is a bimethacryloyl guanidine derivative, which has been shown to specifically inhibit NHE3 activity. In NHE-deficient mouse fibroblast cell line LAP1, the rat NHE3 activity is inhibited by S3226 with K_i of 0.23 μM, while the human NHE3 activity is inhibited by S3226 with K_i of 0.02 μM. In the case of NHE2, even 80 μM S3226 showed ~ 50% inhibition on NHE2 activity [30]. In our experiments, NHE8 expressing PS120 cells were treated with S3226 during the NH₄Cl washout phase and the pH_i recovery phase. As shown in Fig. 7A, 20 μM S3226 inhibited ~ 35% of NHE8 activity, while 80 μM S3226 inhibited ~ 80% of NHE8 activity in PS120 cells. These observations suggest that NHE8 is more sensitive to S3226 compared with NHE2.

Effect of PMA on the NHE8 Activity in PS120 Cells

PMA has been shown to stimulate NHE1 and NHE2 activity, and to inhibit NHE3 activity in stably transfected PS120 cells [31]. The effect of PMA on this newly identified NHE8 remain unknown. To test if PMA affect NHE8 activity, NHE8 expressing PS120 cells were

Fig. 7. Effect of S3226 and PMA on the NHE8 Activity. PS120 cells transfected with NHE8 were loaded with 40mM NH₄Cl in HBSS for 5 minutes, and then washed with sodium-free HBSS for 2 minutes before adding sodium-containing HBSS. The rate of pHi recovery was analyzed during the

initial 30 second following addition of sodium-containing HBSS. The relative changes on pHi recovery at 30 second were calculated by dividing ΔpHi from treatment groups by control group. Panel A. Effect of S3226 on NHE8 activity. pHi measurement was measured in the presence or absence of S3226. S3226 was present during the NH₄Cl washout phase and the recovery phase. Data were collected from 15-22 cells. * $p < 0.03$ for 20 μM S3226 v.s. control and 80 μM S3226. ** $p < 0.03$ for 80 μM S3226 v.s. control and 20 μM S3226. Panel B. Effect of PMA on NHE8 activity. pHi measurement was performed in cells with or without prior PMA treatment (100 nM, 60 minutes). Data were collected from 15-22 cells.

treated with 100 nM PMA for 60 minutes before measuring pHi recovery rate after acid load. As shown in Figure 7B, PMA has no significant effect on NHE8 activity in PS120 cells.

Discussion

Recently, we and others cloned a new purported sodium hydrogen exchanger NHE8 from mouse kidney and rat intestine, and demonstrated that this transporter was expressed at the apical membrane of the renal and the intestinal epithelial cells [7, 14]. However, the specific expression pattern of NHE8 gene along the intestinal tract is not known. Moreover, the ability of this protein to transport H⁺ in a Na⁺ dependent manner has not been demonstrated. To better understand the role of NHE8 in the intestinal tract, we explored the distribution of NHE8 mRNA along the gastrointestinal tracts in human and mouse. Our data indicated that NHE8 mRNA is expressed along the entire intestinal tract both in human and mouse. High levels of NHE8 mRNA expression were detected in the jejunum and colon of both human and mouse. However, differences in expression between these species were observed with expression of NHE8 relatively higher in the ileum of adult mice, and stomach of humans, respectively. Moreover, in mouse intestine, the expression of NHE8 was higher in younger mice (2 and 3 weeks old) than adult mice (3 months), which is similar to our previous observation in rat [14]. Since NHE8 appears to

be generally expressed through the alimentary canal, from stomach to colon, it is likely to play an important role in the mammalian digestive system particularly when NHE2 and NHE3 expression are low. The high expression of NHE8 during early life suggests that it plays a role in the transport of electrolyte at this critical period of development. Therefore, the distribution of NHE8 may provide more pathophysiological understanding of infant diarrheal disorders, which accounts for 5 million deaths per year in children below five years of age. It is conceivable that NHE8 could be a target for viral/bacterial agents resulting in diarrhea.

To determine if the NHE8 protein is a functionally active NHE antiporter, and to better understand its physiological roles, the rat NHE8 cDNA was cloned into pcDNA6.2 mammalian lumio gateway vector system and expressed in PS120 cells. PS120 cells are a Na⁺/H⁺ exchanger-deficient derivative of the Chinese hamster lung fibroblast cell line (ATCC # CCL39), thus these cells are widely used to study NHE protein activity and regulation [31-34]. To enhance the number of cells within the population that express the NHE8 construct, PS120 cells were transfected with NHE8 cDNA by Effectene-mediated transfection, followed by acid selection. The vector-only transfected PS120 cells did not survive acid selection, while NHE8 cDNA transfected PS120 cells survived and propagated after acid selection. pHi recovery experiments demonstrated that sodium-dependent hydrogen exchanger activity was significant in NHE8 cDNA transfected PS120 cells. These findings indicate

that PS120 cells can functionally express the rat NHE8 transporter making it possible to study transport characteristics and functional regulation of the rat NHE8 transporter in mammalian cells.

The transporter kinetics mediated by rat NHE8 is different from other intestinal NHEs. Comparing the sodium affinity of rat NHEs, the $K_{m[Na^+]}$ for rat NHE1, NHE2 and NHE3 are 10, 50 and 4.7mM respectively [35, 36], while rat NHE8 demonstrated its sodium affinity at $K_{m[Na^+]}$ of 23mM. These data suggest that NHE8 has relative high sodium affinity than NHE2. Comparing the proton affinity among the rat NHEs, the $K_{m[pH_i]}$ for rat NHE1, NHE2 and NHE3 are at pH6.75, pH6.9, and pH6.45 respectively [35, 36], while rat NHE8 showed a $K_{m[pH_i]}$ at pH6.5. These suggest that rat NHE8 has similar proton affinity as NHE3. Therefore, our kinetic data indicate that NHE8 is indeed different from other rat NHEs.

NHE8 also has unique profiles with respect to inhibition by drugs that have specificity for NHE1-3. HOE 694 is an amiloride analog that is specific inhibitor of NHE1 and NHE2. In PS120 cells, this compound inhibits NHE1 activity with a K_i of 0.16 μ M, whereas NHE2 activity is inhibited with a K_i of 5 μ M. Conversely, NHE3 is relatively insensitive to HOE694 with a K_i of 650 μ M [29]. In the case of NHE8, 1 μ M HOE694 had no effect on its activity, while 10 μ M HOE694 significantly inhibited its activity, suggesting that the sensitivity of NHE8 to HOE694 is similar to NHE2.

S3226 {3-[2-(3-guanidino-2-methyl-3-oxo-propenol)-5-methylphenyl]-N-isopropylidene-2-methyl-acrylamide dihydrochlorid} potently and specifically inhibits rat NHE3 activity with K_i of 0.23 μ M, whereas NHE1 and NHE2 are relatively insensitive to S3226 with K_i of 3.6 μ M and 80 μ M respectively [30]. In our experiments, 80 μ M S3226 inhibited most of the NHE8 activity, suggesting that NHE8 is more sensitive to S3226 compared with NHE2.

The difference in regulation of NHE8 by phorbol ester, PMA, was also studied. PMA has been shown to stimulate NHE1 and NHE2 activity, and to inhibit NHE3 activity through protein kinase C activation in PS120 cells [31]. To further understand the characteristics of NHE8

protein, we tested the role of PMA on NHE8 activity in PS120 cells. Our data showed that PMA treatment (100 nM, 60 minute) had no effect on NHE8 activity, which suggested that NHE8 is unresponsive to PMA. The insensitivity to PMA suggests that NHE8 is unlikely subject to protein kinase C regulation, unlike other rat NHE1, 2 and 3 in PS120 cells.

In summary, we determined the gastrointestinal segmental distribution of NHE8 expression, and characterized NHE8 transporter activity in NHE8-construct transfected PS120 cells. The NHE8 gene is differentially expressed along the gastrointestinal tracts of human and mouse from stomach to colon, also with some minor differences in expression levels between mouse and human. Relations between regional differences in mRNA expression and function remain to be determined. We also demonstrated that rat NHE8 can be functionally expressed in PS120 cells. The expressed NHE8 protein mediates sodium dependent proton exchange with a K_m for sodium of 23mM and K_m for pH_i of 6.5. NHE8 is sensitive to NHE2 inhibitor HOE694, and NHE3 inhibitor S3226. Unlike NHE1, NHE2 and NHE3, NHE8 is not subject to the regulation by protein kinase C activation induced by PMA in PS120 cells. Furthermore, expression of the NHE8 protein, particularly in stomach, is highest in neonatal mice. From these data, we conclude that the NHE8 is a functional sodium-hydrogen exchanger, with inhibitor sensitivity and protein kinase C regulation differing from NHE2 and NHE3. Moreover, our observations strongly support the hypothesis that NHE8 is a functional exchanger that plays an important role in the process of sodium absorption during early life.

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