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Transient Expression of Na^+/H^+ Exchanger Isoform NHE-2 in LLC-PK_1 Cells: Inhibition of Endogenous NHE-3 and Regulation by Hypertonicity

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Abstract. Na^+/H^+ exchanger isoforms NHE-2 and NHE-3 demonstrate distinct tissue expression patterns in renal epithelial cells. NHE-2 is predominantly expressed in the inner medulla whereas NHE-3 is highly expressed in the proximal tubule cells. The purpose of the current experiments was to study the characteristics of NHE-2 upon its own expression in cultured proximal tubule cells, LLC-PK_1. Toward this end, LLC-PK_1 cells were subjected to six cycles of proton suicide. The mutant cells, when grown to confluence and assayed for Na^+/H^+ exchanger by $^{22}Na^+$ influx, showed significant reduction in NHE activity as compared to the parent cells (10.4 nmole/mg prot/4 min in parent cells *vs.* 1.8 in mutant cells, $P < 0.001$, $n = 4$). This remaining exchanger activity was mostly mediated via NHE-3 as shown by inhibition of the Na influx following PKC stimulation (65% with PMA *vs.* 100% without PMA. $P < 0.05$, $n = 4$). The mutant cells were transiently transfected with a pCMV/NHE-2 expression vector using calcium phosphate precipitation method. Northern blot analysis showed the expression of a 3.4 kb transcript only in the transfected cells. The expression peaked at 48 hr and diminished by 96 hr. The exchanger activity at 48 hr after transfection was mostly due to NHE-3 (as shown by inhibition in the presence of PMA) but was significantly lower than in sham transfected cells (1.2 nmoles/mg prot. in NHE-2-transfected and 2.1 in sham-transfected, $P < 0.05$, $n = 4$). At 60 hr after transfection, the cells exhibited PMA-stimulated Na influx (>28%) indicating functional expression of NHE-2. Increasing the osmolality of the media to 510 mOsm/l stimulated the Na^+/H^+ exchanger in NHE-2 transfected cells but inhibited the exchanger activity in sham transfected cells. In conclusion, NHE-2 appears as a 3.4 kb transcript in transfected LLC-PK_1 cells and functional expression of NHE-2 is preceded by inhibition of endogenous NHE-3 activity. The NHE-2 is stimulated by hypertonicity, indicating a likely role for this isoform in cell volume regulation.

Key words: Na^+/H^+ exchange — NHE-2 — NHE-3 — Transfection — Hypertonicity

Introduction

The Na^+/H^+ exchanger isoforms NHE-2 and NHE-3 display distinct cell distribution patterns in mammalian kidneys. In the medulla, NHE-2 mRNA expression is significantly higher than NHE-3 (Tse, 1992, 1993*a*). In the cortex, however, the expression of NHE-2 mRNA is much less than NHE-3 mRNA (Tse, 1992, 1993*a*). The cells which express NHE-2 mRNA in the medulla are epithelial cells of the inner medullary collecting duct (IMCD) (Soleimani, 1994*a*). Northern hybridization studies in IMCD cells have revealed lack of NHE-3 expression (Soleimani, 1994*a*). The majority of NHE-3 expressed in the cortex is found in proximal tubule epithelial cells (Biemesderfer, 1993; Soleimani, 1994*b,c*). Recent experiments indicate that NHE-2 in inner medullary collecting duct cells is localized to the basolateral membranes, whereas NHE-3 in proximal tubules cells is targeted to the luminal membranes (Biemesderfer, 1993; Soleimani, 1994*a,c*). The distinct distribution and localization of NHE-2 and NHE-3 in renal epithelial cells suggests that the two isoforms perform different functions. NHE-3, based on its location in the luminal membranes of proximal tubule cells, is likely to be involved with vectorial transport of Na^+ and bicarbonate. NHE-2, based on its location in the basolateral membranes of medullary collecting duct cells (Soleimani, 1994*b*), is likely involved in cell volume rectification. Indeed, studies in cultured inner medullary col-

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lecting duct cells indicate that long term hypertonicity increases NHE-2 activity and mRNA expression (Soleimani, 1994*a*). NHE-3, however, shows inhibition in response to hypertonicity (Soleimani, 1994*b;* Kapus et al., 1994). In addition, NHE-3 and NHE-2 show differential regulation with respect to PKC stimulation (Tse, 1993*b;* Soleimani, 1995*b*).

A recent study in a rabbit proximal tubule cell line showed that NHE-2 in the proximal tubule cells is likely localized to the luminal membranes (Mrkic et al., 1993). Whether membrane targeting of NHE-2 is cell or tissue specific has not been examined. Moreover, whether regulation of NHE-2 is cell-type specific remains unknown. Several investigations, utilizing transfection approaches, have examined the regulation of NHE-2 and NHE-3 isoforms in Na^+/H^+ exchanger deficient *nonepithelial* cells (Orlowski, 1993; Tse, 1993; Levine, 1993; Kapus, 1994). However, regulation of these isoforms in *epithelial* cells transfected with NHE cDNAs has not been examined. It is likely that NHE-3 regulation may be cell-type specific since NHE-3 is an epithelial specific isoform which is not expressed in nonepithelial cells (NHE-2 is also highly expressed in epithelial cells) (Orlowski, 1992; Tse, 1992, 1993*a;* Wang, 1993). As such, post transcriptional or translational regulation of these exchangers may vary when expressed in nonepithelial cell lines. Moreover, the interaction of NHE-2 and NHE-3 remains unknown as no studies have identified these two isoforms in the same cell line. The purpose of the current experiments was to study the effect of NHE-2 when expressed in LLC-PK$_1$ cells, a proximal tubule cell line. Toward this end, LLC-PK$_1$ cells were subjected to proton suicide and utilized for transient transfection with NHE-2 cDNA. The results indicated that NHE-2 appears as a 3.4 kb transcript in transfected LLC-PK$_1$ cells and that increasing the osmolality stimulated the activity of NHE-2. The results further demonstrated that functional expression of NHE-2 is preceded by inhibition of endogenous NHE-3 activity.

Materials and Methods

Cell Culture

LLC-PK$_1$ cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DMEM/F12) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultured cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was replaced every other day.

Selection of Mutants Underexpressing Na^+/H^+ Exchanger

Subconfluent cultures of LLC-PK$_1$ cells were treated for 16 hr with ethylmethylsulfonic acid (EMS) at 500 μg/ml. The cells were rinsed with phosphate buffered saline, grown in regular medium to confluence, trypsinized, and centrifuged at room temperature. The cells were then subjected to a proton suicide protocol as described (Pouyssegur et al., 1984). Briefly, the cells were incubated for 90 min in a solution that consisted of (in mM): 130 Li-Cl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 5 glucose, 20 HEPES-Tris, pH 7.4. This procedure results in lithium loading of the cells. Thereafter, the cells were pelleted, washed, incubated for 120 min at 37°C in a solution that consisted of (in mM): 130 choline chloride, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, and 20 of MES (2-N-morpholino-ethane sulfonic acid) at pH 5.5. This step results in acid loading secondary to exchanging internal Li^+ for external H^+. The extreme intracellular acidosis results in death of a majority of the cells. The cells were then centrifuged, recovered, and seeded to culture-grade plastic dishes in DMEM/F12 medium (pH 7.6) for 10 days. Cells underexpressing or deficient in the Na^+/H^+ exchanger survive the proton suicide procedure. Cells were trypsinized and subjected while in suspension to five more suicide cycles. The cells were then subcultured and passaged at very high dilutions (1:1000) to isolate individual colonies. A number of individual colonies were isolated with cloning cylinders and finally collected and subcultured. The Na^+/H^+ exchanger activity was analyzed following each suicide cycle by measuring $^{22}Na^+$ uptake.

Transient Transfection with NHE-2 cDNA

Mutant LLC-PK$_1$ cells were transfected with a full length rat NHE-2 cDNA vector by the calcium phosphate-DNA coprecipitate method (Graham & Van der Eb, 1973). In brief, cells were grown in 100 mm dishes and then transfected with pCMV/NHE-2 (15 μg) plasmid by the calcium phosphate-DNA precipitate technique. Cells were subjected to 15% glycerol shock 18 hr after transfection as described (Parker & Stark, 1979). Transfected cells were then grown in normal media. For influx experiments, the cells were grown in 24-well plates following glycerol shock. For RNA preparation, the cells were grown in 100 mm dishes. For sham control, cells were incubated with calcium phosphate (no DNA) and subjected to 15% glycerol shock.

Measurement of the Na^+/H^+ Exchanger Activity

$^{22}Na^+$ Influx

Uptake of radiolabeled sodium, by both parent LLC-PK$_1$ and by mutant LLC-PK$_1$ cells, was measured as previously described (Orlowski, 1993; Soleimani, 1994*b*). Cells were washed three times with a Na-free buffer consisting of (in mM): 140 chloride salt of NMDG (N-methyl-D-glucamine), 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 7 HEPES, pH 7.4 (solution A). The cells were then incubated in an ammonium-containing solution consisting of (in mM): 110 chloride salt of NMDG, 30 NH_4Cl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 7 HEPES, pH 7.4 (solution B) for 10 mins. Thereafter, the ammonium-containing solution was replaced with uptake solution (solution A) containing 2 mM $^{22}NaCl$. The $^{22}Na^+$ uptake reaction was stopped after 4 min using four rapid washes with ice-cold saline. Cell-associated radioactivity was extracted with 1 ml of 1 N sodium hydroxide and counted by scintillation spectroscopy.

Na-Dependent Acid Extrusion

The intracellular pH in parent or mutant LLC-PK$_1$ cells grown on coverslips was measured with the use of pH-sensitive dye 2′,7′-biscarboxyethyl-5 (and -6) carboxyfluorescein (BCECF) as employed before (Soleimani, 1994*a;* Kapus, 1994; Soleimani, 1995*a*). cells were incubated in the presence of 5 μM BCECF for 15 min in a solution

consisting of (in mM): 140 NaCl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 7 HEPES, pH 7.4 (solution C). The fluorescence of BCECF was monitored in a thermoregulated (37°C) double excitation beam spectrofluorometer (PTI double-beam fluorometer, Delta Scan I, Photon Tech, South Brunswick, NJ). A calibration curve was generated using KCl/nigericin and solutions of vary pH. The fluorescence ratio at excitation wavelengths of 500 and 450 nm was utilized to determine intracellular pH values in the experimental groups by comparison to the calibration curve. Acid loading of cells grown on coverslips was achieved via NH_4 pulse using an NH_4-containing solution (solution B). Cell acidification was induced by replacing the NH_4Cl solution with a sodium-free solution (solution A). Following intracellular acidosis, the initial rate of pH_i recovery was monitored in a sodium-containing solution (solution C).

Isolation of Total and Poly(A)$^+$ RNA

Total cellular RNA was extracted from the cells by the method of Chomczynski and Sacchi. Cells were scraped off the plates with homogenizing buffer consisting of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercapto-ethanol. Protein was extracted by phenol:chloroform (1:1) and RNA was precipitated by isopropanol (Chomczynski & Sacchi, 1987). Poly(A)$^+$ RNA was extracted by passing total cellular RNA twice through oligo dT cellulose spin columns (5′Prime-3′Prime, Boulder, CO). Total and poly(A)$^+$ RNA were quantitated spectrophotometrically at 260 nm.

Northern Hybridization

Poly(A)$^+$ RNA samples, 10–15 μg/lane, were loaded and fractionated on 1% agarose-formaldehyde gels and transferred to nylon membranes by capillary diffusion (Thomas). The membranes were baked at 80°C for 2 hr. The integrity of RNA preparations and the extent of RNA transfer was estimated by viewing the ethidium bromide stained gels and membranes. The membranes were prehybridized for 6 hr at 42°C in 50% formamide, 5 × SSPE, 5 × Denhart's solution, 0.5% SDS, and 200 μg of denatured salmon sperm DNA/ml. The hybridization was carried out overnight at 42°C in the presence of 30–50 × 10^6 cpm ^{32}P-labeled DNA probe for NHE-2 or β-actin. The cDNAs were labeled with ^{32}P-labeled deoxynucleotides using a random primed DNA labeling kit (5 Prime-3 Prime). Following hybridization, the membranes were washed twice in 2 × SSC, 0.5% SDS for 30 min at room temperature, and once for 30 min at 60°C in 0.1 × SSC, 0.5% SDS. For NHE2 the membranes were given an additional wash at 50°C for 30 min in 2 × SSC, 0.5% SDS. Membranes were exposed to Kodak X-Omat film at −70°C with intensifying screens for 72–120 hr. For NHE-2, the AvaI-AvaI fragment (nucleotides 174-2032) was used for hybridization.

Protein Measurements

Protein concentration was determined by the bicinchoninic acid (BCA) method according to the manufacturer's protocol (Pierce Company, Rockford, IL).

Materials

^{22}Na and ^{32}P were purchased from New England Nuclear (Boston, MA). Dimethylamiloride (DMA), Nitrocellulose filters, and other chemicals were purchased from Sigma Chemical (St. Louis, MO). BCECF and nigericin were purchased from Molecular Probes (Eugene, OR). Oligo dT cellulose spin columns and Random Primed DNA labeling kit were purchased from 5′Prime-3′Prime.

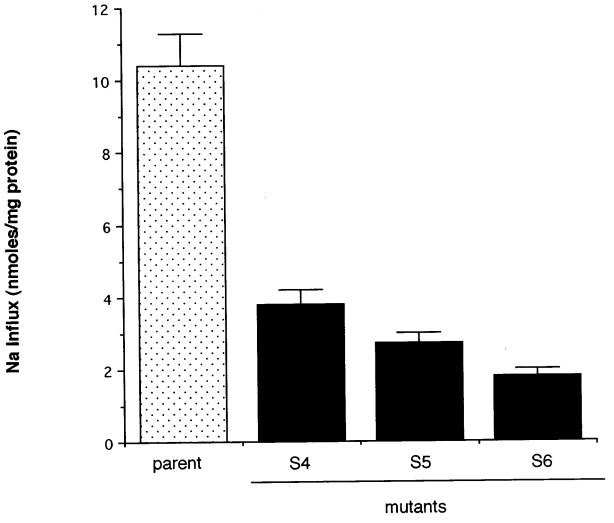

Fig. 1. Acid-stimulated, DMA-sensitive $^{22}Na^+$ influx. Parent cells (left lane) or mutant LLC-PK$_1$ cells from the 4th, 5th, and 6th proton suicide cycles (S4, S5 and S6, respectively) were group to confluence on plastic dishes. Acid-stimulated $^{22}Na^+$ influx was measured at 4 min in the presence or absence of 0.5 mM dimethylamiloride (DMA). Values represent mean ± SE for four experiments performed in quadruplicate.

Statistical Analysis

The data are expressed as mean ± SE. Statistical analysis was determined using analysis of variance or ANOVA where appropriate. $P < 0.05$ was considered statistically significant.

Results

$^{22}Na^+$ Influx

In the first series of experiments we measured the Na^+/H^+ exchange activity in parent and mutant LLC-PK$_1$ cells by $^{22}Na^+$ influx. Cells from the 4th, 5th, and 6th cycles of proton suicide were grown in 24-well plastic dishes, acid loaded by ammonium prepulse, and influx of $^{22}Na^+$ was assayed at 4 min in the presence or absence of 0.5 mM dimethylamiloride (DMA). As illustrated in Fig. 1, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was significantly decreased in cells subjected to proton suicide. The activity of the exchanger in cells from the 6th proton suicide cycle was only 13% of the activity in parent LLC-PK$_1$ cells. The results further indicated that the bulk of exchanger activity was diminished in the first 3 cycles. Uptake studies demonstrated that proton suicide cycles beyond the 6th cycle did not diminish the exchanger activity further (*data not shown*). We therefore

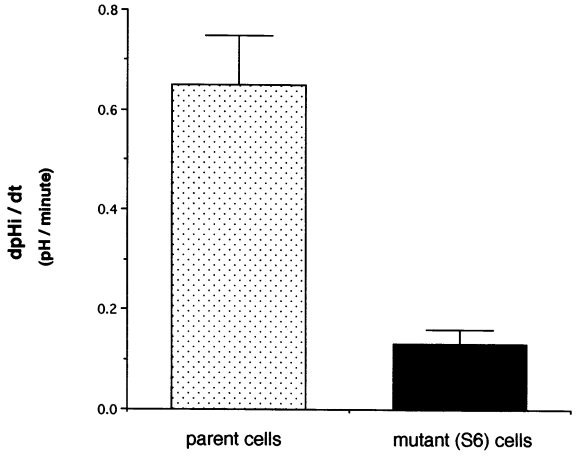

Fig. 2. Sodium-dependent pH_i recovery. Parent Cells or mutant cells from the 6th suicide cycle were grown on coverslips, pulsed with ammonium, and assayed for Na^+/H^+ exchanger activity as sodium-dependent H^+ extrusion. Values represent mean ± SE for five coverslips.

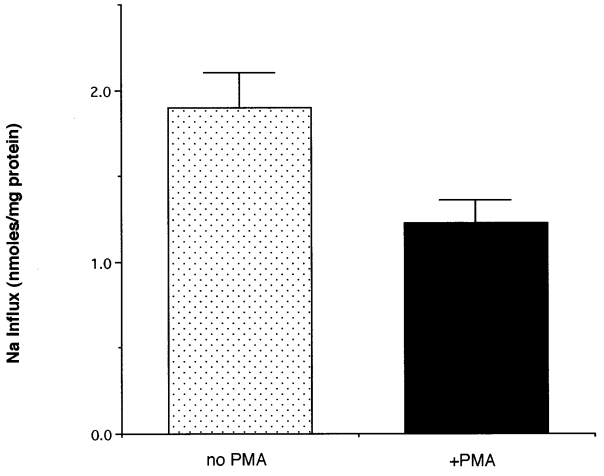

Fig. 3. Effect of PMA on Na^+/H^+ exchanger activity. Mutant (S6) cells from the 6th suicide cycle were grown to confluence in plastic dishes, pulsed with ammonium, and incubated with a Na-free, PMA-containing media. $^{22}Na^+$ influx was measured at 4 min ± 0.5 mM DMA. Values represent mean ± SE for three experiments in quadruplicate.

used the cells from the 6th proton suicide cycle (called S6 cells) for the rest of the experiments.

Na-dependent Acid Extrusion

The pH-sensitive dye, BCECF, was used to compare Na^+/H^+ exchanger activities in parent and mutant LLC-PK$_1$ cells. Cells from the 6th cycle of proton suicide (S6 cells) were grown to confluence on coverslips and assayed for Na-dependent pH_i recovery from an acid load. As shown in Fig. 2, Na-dependent pH_i recovery was significantly decreased in mutant cells as compared to parent cells. As shown, the initial pH_i recovery from an acid load (dpH_i/dt) was 0.65 ± 0.09 pH/min in parent and 0.13 ± 0.03 pH/min in mutant LLC-PK$_1$ cells, $P < 0.01$. Steady state pH_i was 7.44 ± 0.06 in parent cells and 7.37 ± 0.05 in mutant cells ($P > 0.05$, $n = 5$ coverslips for each group). Following acid loading with NH_4Cl, intracellular pH decreased to 6.47 ± 0.07 in parent cells and 6.41 ± 0.06 in mutant cells ($P > 0.05$, $n = 5$ coverslips for each group).

Effect of PKC Stimulation on Exchanger Activity

LLC-PK$_1$ cells express NHE-3 and NHE-1 on their luminal and basolateral membrane domains, respectively (Haggerty et al., 1988; Casavolo et al., 1992; Reilly, 1991; Soleimani, 1994*b*). NHE-3 and NHE-1 are shown to be differentially regulated by PKC; NHE-3 is inhibited whereas NHE-1 is stimulated by PKC (Tse, 1993*b;* Soleimani, 1995*b*). In the next series of experiments, we examined whether proton suicide had depleted the mutant cells of their NHE-3. Toward this end, S6 cells were grown to confluence in plastic dishes, pulsed with ammonium, and incubated with a Na-free medium that contained PMA (phorbol 12-myristate 13-acetate), at 10^{-7} M. After 5 min, the solution was replaced with an identical medium that contained 2 mM $^{22}Na^+$ and the uptake was measured at 4 min ± 0.5 mM DMA. As demonstrated in Fig. 3, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was significantly decreased in mutant cells incubated with PMA. These results indicate that the residual exchanger activity in mutant LLC-PK$_1$ cells is to a large extent mediated via NHE-3.

Limited DMA Dose-Response Inhibition

NHE-3 is relatively resistant whereas NHE-1 is highly sensitive to inhibition by potent amiloride analogues (Haggerty et al., 1988; Orlowski, 1993; Soleimani, 1995*b*). To further characterize the Na^+/H^+ exchanger in mutant cells, a limited dose-response inhibition of the exchanger activity by DMA was performed. S6 Cells were grown to confluence in plastic dishes, pulsed with ammonium, and assayed for $^{22}Na^+$ influx in the presence of 0.5 and 5 μM DMA. As shown Fig. 4, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was relatively resistant to DMA, further indicating that mutant cells predominantly express NHE-3.

Transient Expression of NHE-2

The purpose of the next series of experiments was to express NHE-2 in mutant LLC-PK$_1$ cells. Accordingly, S6 mutant cells were grown in 100-mm dishes and transfected with a full length rat NHE-2 cDNA vector using a

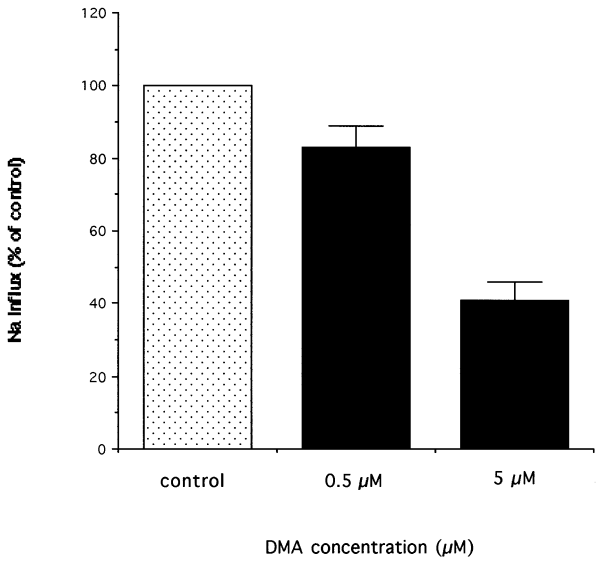

Fig. 4. Limited dose response inhibition of Na^+/H^+ exchanger by DMA. Mutant (S6) cells from the 6th suicide cycle were grown to confluence in plastic dishes, pulsed with ammonium, and assayed for $^{22}Na^+$ influx in the presence of 0.5, and 5 μM DMA. Values represent mean ± SE for three experiments performed in quadruplicate.

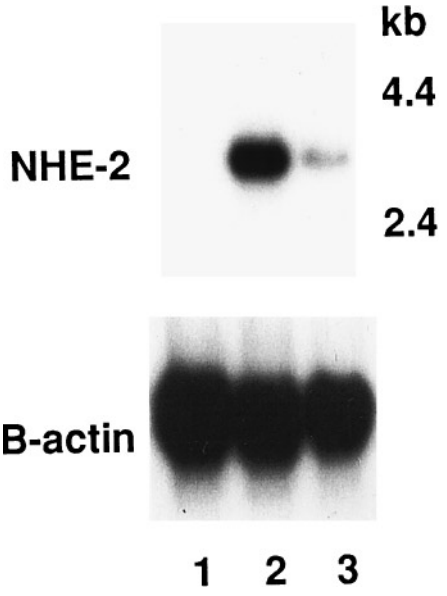

Fig. 5. Transient expression of NHE-2 in mutant LLC-PK$_1$ cells. Representative Northern blot of poly-A^+ RNA isolated from mutant LLC-PK$_1$ cells (lane 1) and mutant cells transfected with NHE-2 cDNA at 48 hr and 96 hr. β-actin transcript levels are shown for comparison. NHE-1 transcript size was 3.4 kb. 20 μg Poly-A^+ RNA were loaded on each lane.

calcium phosphate method (Graham et al., 1973). Cells were subjected to 15% glycerol shock 18 hr after transfection. The cells were then grown in normal media in 100-mm dishes. For sham control, cells were incubated with calcium phosphate (no DNA) and subjected to 15% glycerol shock. Sham and NHE-2 transfected mutant cells were then assessed for expression of NHE-2 mRNA. Poly$(A)^+$ RNA was isolated from mutant control (lane 1), and NHE-2 transfected cells at 48 h (lane 2) and 96 hr (lane 3) after transfection. Poly$(A)^+$ RNA was size fractionated, transferred to a nylon membrane, and probed with radiolabeled NHE-2 cDNA. A representative Northern hybridization is shown in Fig. 5. As shown, a 3.4 kb transcript was only present in NHE-2 transfected cells (lanes 2 and 3), indicating expression of NHE-2 in mutant LLC-PK$_1$ cells. The sham-transfected cells did not express NHE-2 mRNA, consistent with absence of endogenous NHE-2 in these cells. Northern hybridization in parent LLC-PK$_1$ cells also did not reveal any NHE-2 mRNA (*data not shown*). The expression of NHE-2 mRNA peaked at 48 hr and was significantly diminished at 96 hr (lanes 2 and 3).

$^{22}Na^+$ Influx in Control and NHE-2 Transfected Mutant Cells

To assess functional expression of NHE-2, sham- and NHE-2-transfected cells were grown in 24-well plates and assayed for exchanger activity by $^{22}Na^+$ influx. As indicated in Fig. 6, left two bars, the DMA-sensitive $^{22}Na^+$ influx at 48 hr after transfection was significantly decreased in NHE-2-transfected cells compared to sham-transfected cells. The exchanger activity in control and NHE-2-transfected cells were comparably inhibited in the presence of PMA (38% in control and 32% in NHE-2-transfected cells, *data not shown*). These results are consistent with decreased endogenous NHE-3 exchanger activity in NHE-2-transfected cells. In the next series of experiments, we assessed functional expression of NHE-2 in mutant cells at 60 hr after transfection. As shown in Fig. 6, right two bars, the DMA-sensitive $^{22}Na^+$ influx at 60 hr after transfection was significantly increased in NHE-2-transfected cells compared to sham-transfected cells ($P < 0.05$). The intracellular pH_i following acid loading was comparable in sham- and NHE-2-transfected cells at 48 and 60 hr (6.38 ± 0.04 *vs.* 6.41 ± 0.05 in sham transfectants at 48 and 60 hr, and 6.41 ± 0.05 *vs.* 6.44 ± 0.04 in NHE-2 transfectant at 48 and 60 hr, respectively, $n = 4$ coverslips for each group).

Effect of PMA on Exchanger Activity in NHE-2 Transfected Cells

To characterize the NHE-2 exchanger further, the effect of PKC activation on the exchanger activity was assessed in mutant cells at 60 hr after transfection. NHE-2-transfected cells were grown to confluence in plastic dishes and assayed for $^{22}Na^+$ influx in the presence of

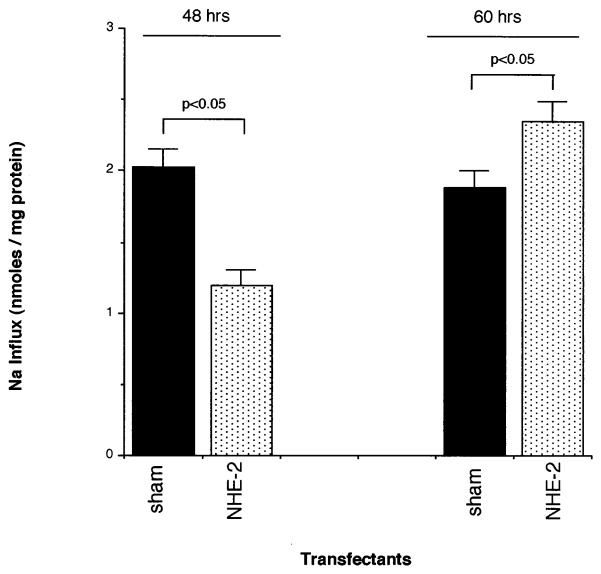

Fig. 6. Acid-stimulated, DMA-sensitive $^{22}Na^+$ influx in NHE-2 transfected mutant cells. Mutant LLC-PK$_1$ cells from the 6th proton suicide cycle were transiently transfected and assayed either at 48 hr (left panel, sham transfected or transfected with NHE-2 cDNA) or at 60 hr (right panel, sham transfected or transfected with NHE-2 cDNA). Acid-stimulated $^{22}Na^+$ influx was measured at 4 min ± 0.5 mM DMA. Values represent mean ± SE for three experiments performed in quadruplicate.

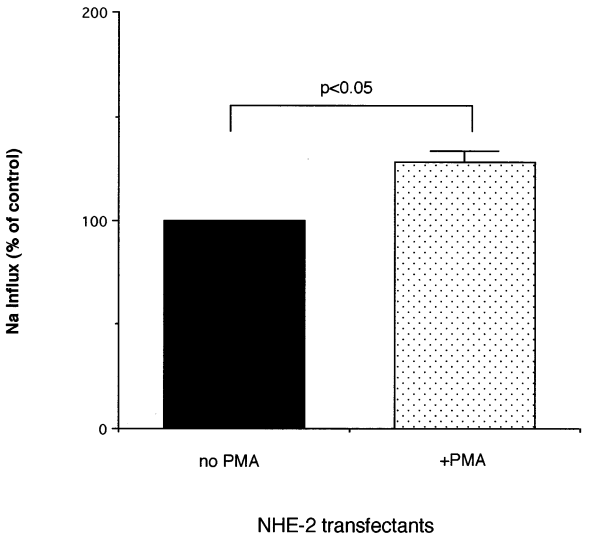

Fig. 7. Effect of PMA on Na^+/H^+ exchanger activity in NHE-2 transfected cells. Cells were subjected to transfection protocol, grown to confluence in plastic dishes, pulsed with ammonium, and incubated with a Na-free, PMA-containing media (at 60 hr after transfection). $^{22}Na^+$ influx was measured at 4 min ± 0.5 mM DMA. Left lane: NHE-2 transfected cells at 60 hr after transfection with no PMA. Right lane: NHE-2 transfected cells at 60 hr after transfection with PMA. Values represent mean ± SE for three experiments performed in quadruplicate.

PMA (10^{-7} M) in a manner similar to Fig. 3. As demonstrated in Fig. 7, acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake was significantly increased in the presence of PMA in NHE-2-transfected cells at 60 hr after transfection ($P < 0.05$).

Effect of Hypertonicity on Exchanger Activity in NHE-2-Transfected Cells

To examine regulation of the NHE-2 exchanger by hypertonicity, acid-stimulated $^{22}Na^+$ influx was assessed in the presence of isotonic (310 mOsm) or hypertonic medium (510 mOsm) at 60 hr after transfection. Cells were equilibrated at pH_i 6.3 and were then exposed to either isotonic medium (310 mOsm/l) or hypertonic medium (510 mOsm/l) as described (Soleimani, 1994*b*). As indicated in Fig. 8, high osmolality significantly increased the acid-stimulated, DMA-sensitive $^{22}Na^+$ uptake in NHE-2-transfected cells as compared to sham-transfected cells ($P < 0.05$). When compared to $^{22}Na^+$ uptake in isotonic medium, NHE-2 transfectants in hypertonic medium showed increased exchanger activity ($P < 0.05$).

Parent LLC-PK$_1$ cells subjected to transient transfection protocol also showed reduction in endogenous NHE-3 activity at 48 hr after transfection (9.6 ± 0.83 in sham-transfected parent cells *vs.* 6.4 ± 0.73 nmoles/mg protein in NHE-2-transfected parent cells, $P < 0.05$, $n =$ 3). These results indicate that inhibition of NHE-3 activity following NHE-2 transfection is not limited to mutant cells.

Discussion

All Na^+/H^+ exchanger isoforms subserve the same basic function, that is, they extrude acid in exchange for sodium. However, Na^+/H^+ exchanger isoforms display distinct functional features that distinguish these isoforms. The NHE-1 isoform is ubiquitous in its expression and is present in both polar and nonpolar cells (Orlowski, 1992). NHE-2 is expressed in polar cells with limited expression in nonpolar cells (Wang, Orlowski & Shull, 1993; Tse, 1993). NHE-3 is exclusively expressed in polar cells (Orlowski, 1992; Tse, 1992). NHE isoforms display differences with respect to regulation by protein kinase C, calmodulin-dependent kinase, and hypertonicity (Tse, 1993*b;* Soleimani, 1994*b;* Kapus et al., 1994). Recent investigations have characterized certain aspects of NHE isoforms by transfecting NHE-deficient nonpolar cells with corresponding NHE cDNAs. In the present studies, we have transfected renal epithelial cells LLC-PK$_1$ with NHE-2 cDNA. We chose an epithelial cell line as a host for transfection experiments since NHE-2 shows high level of expression in epithelial cells (Wang et al., 1993). It is likely that NHE-2 regulation in polar cells may vary compared to nonpolar cells. More-

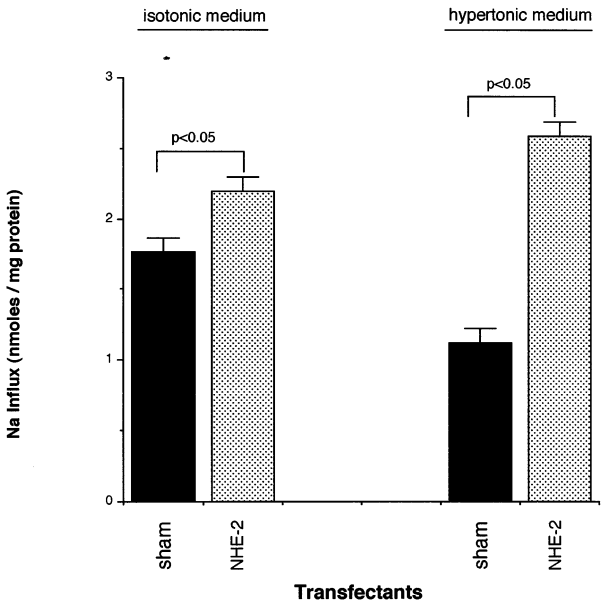

Fig. 8. Effect of hypertonicity on Na^+/H^+ exchanger activity in NHE-2 or sham-transfected cells. Transfected cells (NHE-2 or sham) were grown to confluence in plastic dishes for 60 hr, acidified, and incubated with a Na-free isotonic (310 mOsm) or hypertonic (510 mOsm) medium for 5 min. Thereafter, the 4 min influx of $^{22}Na^+$ influx ± 0.5 mM DMA was measured in isotonic or hypertonic medium. Left two lanes: sham or NHE-2 transfected cells in isotonic medium. Right two Lanes: sham or NHE-2-transfected cells in hypertonic medium. Values represent mean ± SE for four experiments performed in quadruplicate.

over, these studies may allow for studying the interaction of NHE-2 with NHE-3. This latter issue is very important as these two isoforms are coexpressed in a variety of polar tissues including stomach, kidney and brain. However, no study is available regarding the interaction of these two isoforms. LLC-PK$_1$ cells, a cell line with renal proximal tubule properties, express NHE-3 on their luminal membranes (Soleimani, 1994*b*). The experiments shown in Figs. 1–4 demonstrate that subjecting the LLC-PK$_1$ cells to proton suicide resulted in significant reduction in the exchanger activity. The remaining exchanger activity was predominantly mediated via NHE-3. Transient transfection of LLC-PK$_1$ cells with NHE-2 cDNA resulted in the expression of a 3.4 kb transcript, a size appropriate for NHE-2 (Fig. 5). The expression of NHE-2 peaked at 48 hr and significantly diminished at 96 hr. Studies in transfected cells (Fig. 6) demonstrated that functional expression of NHE-2 was preceded by inhibition of endogenous NHE-3 activity. NHE-2 functional activity was apparent at 60 hr after transfection (Fig. 6). The NHE-2 activity increased in response to PKC activation (Fig. 7), further indicating that the transfected NHE-2 is functionally operational.

One interesting feature of the NHE-2 transfected cells was their increased exchanger activity in response to high osmolality (Fig. 8). It has been shown that NHE-3 in LLC-PK$_1$ cells (Soleimani, 1994*b*) or NHE-3-transfected nonepithelial cells (Kapus et al., 1994) is inhibited in response to hypertonicity. Indeed, the sham-transfected cells, which express NHE-3, showed decreased exchanger activity in hypertonic medium, further confirming the inhibitory effect of hypertonicity on this isoform (Fig. 8). NHE-2, however, has been shown to be stimulated by hypertonicity (Kapus et al., 1994). The hypertonicity-induced exchanger stimulation in NHE-2-transfected cells in the current experiments (Fig. 8) suggest that NHE-2 may play a role in cell volume regulation.

Another intriguing feature of these studies was inhibition of NHE-3 activity prior to functional expression of NHE-2. The molecular mechanism of this inhibition remains unknown at the present. Whether expression of NHE-2 inhibited transcriptional, translational, or post-translational processing of endogenous NHE-3 is not clear. Northern hybridization and immunoblot analysis in mutant cells did not reveal any NHE-3 mRNA or protein, likely due to significant reduction in NHE-3 mRNA and protein (*data not shown*). Northern hybridization experiments in parent LLC-PK$_1$ cells (sham transfected *vs.* NHE-2 cDNA transfected), however, did not show any significant differences in NHE-3 mRNA levels (*data not shown*). Another possibility with regard to NHE-3 inhibition might be alterations at post-translational level. It is not clear whether NHE-2 and NHE-3 utilize the same intracellular processing pathway. Whether NHE-2 and NHE-3 are targeted to the same membrane domain (luminal membrane) or directed to opposite membrane domains (i.e., NHE-3 to the lumen and NHE-2 to the basolateral membranes) is not clear. To answer this question, NHE-2 transfected cells were grown on permeable Millicell-HA filters, and assayed for luminal or basolateral exchanger activity using $^{22}Na^+$ influx method. PMA-activated, acid-stimulated $^{22}Na^+$ influx was observed from both membrane surfaces (*data not shown*), indicating that NHE-2 was targeted to both membrane domains. These results, however, have to be interpreted with caution, as these experiments were performed only 60–80 hr after transfection and therefore, the cells did not attain polarity at the time of experiments. The answer to membrane localization of NHE-2 should come from stable transfection of LLC-PK$_1$ cells with NHE-2 cDNA. The targeting of NHE-2 to the luminal or basolateral membrane domain is an intriguing question. Studies in cultured inner medullary collecting duct (Soleimani, 1994*a*) and intestine (Tse, 1993*a*) indicate that NHE-2 is targeted to the basolateral or luminal membranes, respectively. Whether membrane domain targeting of NHE-2 is cell type or tissue specific remains unknown. Those questions could be answered by immunohistochemical staining of renal cells using NHE-2 specific antibodies.

In conclusion, transient expression of NHE-2 resulted in appearance of a 3.4 kb transcript in transfected LLC-PK$_1$ cells. The expression of NHE-2 peaked at 48 hr and diminished at 96 hr. The NHE-2 transfected cells increased their exchanger activity in response to high osmolality. Functional expression of NHE-2 was preceded by inhibition of endogenous NHE-3 activity. Further studies are needed to examine the characteristics of NHE-2 and the molecular mechanism of interaction of NHE-2 and NHE-3 in LLC-PK$_1$ cells.

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