J. Membrane Biol. 190, 117–125 (2002)
DOI: 10.1007/s00232-002-1030-8

The Journal of
Membrane
Biology

Differential Role of Na^+/H^+ Exchange Isoforms NHE-1 and NHE-2 in a Rat Cortical Collecting Duct Cell Line

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Received: 16 April 2002/Revised: 15 July 2002

Abstract. The Na^+/H^+ exchanger (NHE) constitutes a gene family containing several isoforms that display different membrane localization and are involved in specialized functions. Although basolateral NHE-1 activity was described in the cortical collecting duct (CCD), the localization and function of other NHE isoforms is not yet clear, This study examines the expression, localization, and regulation of NHE isoforms in a rat cortical collecting duct cell line ($RCCD_1$) that has previously been shown to be a good model of CCD cells. Present studies demonstrate the presence of NHE-1 and NHE-2 isoforms, but not NHE-3 and NHE-4, in $RCCD_1$ cells. Cell monolayers, grown on permeable filters, were placed on special holders allowing independent access to apical and basolateral compartments. Intracellular pH (pH_i) regulation was spectrofluorometrically studied in basal conditions and after stimulation by NH_4Cl acid load or by a hyperosmotic shock. In order to differentiate the roles of NHE-1 and NHE-2, we have used HOE-694, an inhibitor more selective for NHE-1 than for NHE-2. The results obtained strongly suggest that NHE-1 and NHE-2 are expressed in the basolateral membrane but that they have different roles: NHE-1 is responsible for pH_i recovery after an acid load and NHE-2 is mainly involved in steady-state pH_i and cell volume regulation.

Key words: $RCCD_1$ cell line — Intracellular pH — Na^+/H^+ exchanger — Acid load and hyperosmolality

Introduction

The Na^+/H^+ exchanger (NHE) plays an important role in a variety of cell functions, including intracellular pH regulation (pH_i), cell volume regulation, and transepithelial ion transport [17, 30]. In the kidney, Na^+/H^+ exchange activity is nearly ubiquitous [12, 24, 25, 26] and the expression of four different isoforms has already been described [30, 31, 36, 37]. Immunocytochemical studies have indicated that NHE-1 and NHE-4 have a basolateral distribution [3, 14], whereas NHE-2 and NHE-3 are located along the apical membrane of various nephron segments, including the proximal tubule, loop of Henle and distal convoluted tubule [2, 15]. However, NHE-2 distribution in collecting duct is controversial since some authors propose basolateral localization [33, 34], others apical localization [23], and some studies even propose that NHE-2 is not expressed in the collecting tubule [6, 15].

The physiological role differs among NHE isoforms and it is not yet completely understood. Whereas NHE-1 plays a "house-keeping" role in pH_i and cell volume regulation in many segments of the nephron, the major epithelial isoforms, NHE-2 and NHE-3, are thought to have more specialized roles in regulating Na^+ and water absorption [39, 40]. Another regulatory property of the Na^+/H^+ exchanger is its activation by hyperosmolality, an important mechanism for the control of cell volume. In many cell types acute osmotic shrinkage causes parallel activation of Na^+/H^+ and Cl^-/HCO_3^- exchangers, resulting in net uptake of NaCl and H_2O, which returns cell volume to its original value [21]. Some studies indicate that Na^+/H^+ exchanger isoforms exhibit differential responses to hyperosmolality. It was previously reported that NHE-1, when overexpressed in PS120 fibroblasts, was activated in response to hyperosmotic stress [22, 29]. In contrast, NHE-2 and NHE-3 were found to be inhibited in PS120 fibroblast [29]. Additionally, NHE-2 levels were shown to be increased by hyperosmolarity in mouse inner

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medullary collecting duct cells (mIMCD-3) [33]. Moreover, the NHE-4 isoform was also increased by hyperosmolality in fibroblasts [5]. However, it is not yet known whether hyperosmolality activates NHE in the cortical collecting tubule (CCD) cells.

The aim of the present work was to study the expression, localization, and regulation of Na^+/H^+ exchanger isoforms in a rat cortical collecting duct cell line ($RCCD_1$). These cells were previously used as a good model of CCD to study ion and water transport and their regulatory pathways of this part of the nephron [4, 9, 19]. pH_i studies were performed using fluorescence measurements in monolayers grown on permeable supports, allowing independent and free access to the apical or the basolateral compartments. RT-PCR and Western-blot studies demonstrated the presence of NHE-1 and NHE-2 isoforms in $RCCD_1$ cells. The results obtained strongly suggest that NHE-1 and NHE-2 are expressed in the basolateral membrane, but that they have different roles: NHE-1 would be responsible for pH_i recovery after an acid load and NHE-2 would be mainly involved in steady-state pH_i and cell volume regulation.

Materials and Methods

Cell Culture

$RCCD_1$ cells grown in flasks were, once a week, routinely passed using trypsin [4]. The defined culture medium was 1:1 Ham's F-12/DMEM; 14 mM $NaHCO_3$, 3.2 mM glutamine; 5×10^{-8} M dexamethasone; 3×10^{-8} M sodium selenite; 5 μg/ml insulin; 10 μg/ml epidermal growth factor; 5×10^{-8} M triodothyronine; 10 units/ml penicillin-streptomycin; 20 mM HEPES (pH 7.4) and 2% fetal bovine serum (Gibco BRL). For experiments, cells between passages 20 and 45 were seeded on Transwell holders (3-μm pore "tissue culture treated" Nucleopore filters; 4.5 cm^2 surface area; Costar Corp.) and cultured during six or seven days.

Solutions

All pH solutions were adjusted to 7.4 using Tris-[hydroxymethyl] aminomethane; compositions are summarized in Table 1. Solution osmolalities were measured in a pressure vapor osmometer (VAPRO). In some experiments the inhibitors ethylisopropylamiloride (EIPA) and HOE-694 (a benzoylguanidine derivative) were used.

Intracellular pH Studies

$RCCD_1$ monolayers were placed on a special holder. Briefly, Nucleopore filters containing the cells were inserted between two lucite frames, separating two fluid compartments when diagonally placed in a quartz cuvette. Free access both to the apical and basolateral baths was possible. Measurements were made with a computerized and thermoregulated (37°C) spectrofluorometer (Jasco 770, Japan). The cell monolayer was placed for measurements forming a 45° angle with the exploring beam. Fluorescence emission was monitored at 535 nm, with excitation wavelengths of 439 and 510 nm.

Table 1. Composition of solutions used for determination of pH_i recovery

Solution	A	B	C	D
NaCl	145	–	127	139
$NaHCO_3$	–	–	–	10
HEPES	30	30	30	20
KCl	3	3	3	5
$CaCl_2$	1.8	1.8	1.8	1
$MgCl_2$	–	–	–	1
$MgSO_4$	1	1	1	0.8
KH_2PO_4	1	1	1	–
K_2SO_4	1	1	1	–
NH_4Cl	–	–	20	–
TMA	–	147	–	–
Glucose	5.6	5.6	5.6	5.6

All concentrations are given in mM; pH was adjusted to 7.4 at 37°C.

For pH_i measurements, the cells were loaded with 8 μM of 2′, 7′ bis(2-carboxyethyl)-5-(and-6) carboxyfluorescein acetoxymethyl ester (BCECF/AM) for 60 min at 37°C, both from the apical and basolateral sides. The ratio of the BCECF fluorescence emitted from dye-loaded cells was calibrated in terms of pH, by incubating the cells in "high-K^+ solution" (in mM: 140 KCl, 4.6 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 5 glucose) and then by permeabilizing the cells with 5 μM nigericin to balance extracellular pH (pH_o) with intracellular pH (pH_i). Then the pH-bathing solution was stepped between pH 6.6 and 8.5. The 510/439 ratio was linear over this pH range ($r = 0.99$, $n = 6$).

In some experiments pH_i regulation was investigated by monitoring pH_i recovery after an acid load by the NH_4Cl-prepulse technique as previously described [7].

Measurement of Water Osmotic Fluxes

To study water osmotic fluxes (J_w), the transwell filters were directly inserted between two-barrel lucite hemi-chambers so as to define two independent compartments, as previously described [9]. One of them (basolateral) was open to the atmosphere, while the other (apical) was hermetically sealed. The closed chamber was connected with a small-diameter polyethylene tube to the net water-measurement system where the net water flux (J_w) was recorded every minute, as previously described [20].

RT-PCR Studies

Total RNA from rat kidney (positive control) or $RCCD_1$ cells were isolated using the SV total RNA Isolation System (Promega). Reverse transcription was performed on 2 μg of total RNA using M-MLV reverse transcriptase (Promega). RNAs were placed in 50 μl of "RT reaction buffer" containing: 1 × M-MLV reverse transcriptase buffer (Promega), 0.5 μg oligo-dt primer, and 10 U/μl RNAsin. The reaction was heated 3 min at 80°C and cooled at 45°C. PCR buffer (25 μl) containing: 1 × M-MLV reverse transcriptase buffer, 2.5 mM $MgCl_2$, 400 μM dNTPs and 400 units M-MLV, were added to half of the reaction. Control experiments, in absence of the enzyme M-MLV, were performed on the remaining 25 μl. RT reaction was carried out for 1 hr at 45°C and stopped by heating 2 min at 95°C.

PCR experiments (30 sec at 94°C, 30 sec at 65°C, 45 sec at 72°C for 35 cycles) were done with 5 μl of the RT reaction using 25 pmol of specific primers for rat NHE-1, NHE-2, NHE-3 and NHE-4 (Table 2). Internal positive control was included in each experiment

Table 2. Specific rat primers used to detect the expression of NHE isoforms

	Primers	Amplified fragment size (bp)	cDNA sequence location (nt)	Data Bank accession number
NHE-1	5′-TGA CCG CAA TTT GAC CAA CTT ATA C-3′	418	219–243	G1280490
	5′-ACA GGA GGC CAC CCA GGA AGA AAG-3′		637–614	
NHE-2	5′-CAA GTT GCC CAC GAT TGT GC-3′	708	309–328	G205318
	5′-GGC TGT GAT CGC CAT GAT GC-3′		1017–998	
NHE-3	5′-TGC CTG GAC ATC CAG TCC TTG G-3′	716	1777–1798	G205706
	5′-CAT GTG TGT GGA CTC AGG GGA AGC-3′		2470–2493	
NHE-4	5′-GGC TGG GAT TGA AGA TGT ATG T-3′	501	1972–1994	G205708
	5′-GCT GGC TGA GGA TTG CTG TAA-3′		2451–2430	

using β-actin-specific primers (sense: 5′ CGG AAC CGC TCA TTG CC 3′; antisense: 5′ ACC CAC ACT GTG CCC ATC TA 3′).

Immunoblotting Studies

Confluent $RCCD_1$ cells were washed three times in cold PBS and were incubated for 30 min in a cold (4°C) lysis buffer containing (in mM) 150 NaCl, 20 Tris, 5 EDTA, 1 phenyl-methylsulfonyl fluoride, 1% Triton 100X, 10 μg/ml antipaine, 10 μg/ml leupeptine and 10 μg/ml pepstatine. The obtained lysate was clarified by centrifugation at 12,000 × *g* for 10 min. The homogenates were subjected to 7.5% SDS-polyacrylamide minigel electrophoresis and transferred to nitrocellulose sheets (Bio-Rad Mini Protean II). Blots were blocked with 5% milk in PBS-T (in mM 80 Na_2HPO_4, 20 NaH_2PO_4, 100 NaCl, and 0.1 % Tween 20, pH 7.5) for 1 hr and incubated with purified rabbit anti-rat NHE-1 (diluted 1:2,000; Alpha Diagnostics International, Cat. N°: NHE11-A) or rabbit anti-rat NHE-2 (diluted 1:2,000; Alpha Diagnostics International, Cat. N°: NHE21-A). The blots were then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin and bands were detected by the enhanced chemoluminescence detection system (BCL, Amersham). Autoradiographs were obtained by exposing nitrocellulose to Kodak XAR film.

Materials

BCECF-AM was purchased from Molecular Probes (Eugene, OR). HOE-694 was kindly provided by Dr. Juergen Puenter (Aventis Pharma Deutschland GmbH, Frankfurt, Germany). All other substances were obtained from Sigma.

Results

Na^+/H^+ Exchanger Isoform Expression in $RCCD_1$ Cells

RT-PCR experiments were performed using specific primers for the rat NHE isoforms that are normally expressed in kidney [17, 30]. Assays were undertaken using total RNA extracted from $RCCD_1$ cells and rat kidneys (used as positive controls). Figure 1*A* shows the result of a representative RT-PCR experiment using primers for NHE-1 and NHE-2 isoforms (Table 2). A positive band of ~418 bp for NHE-1 and ~708 bp for NHE-2 were found in kidneys and in $RCCD_1$ cells (three to four different experiments). Figure 1*B* summarizes experiments where specific primers for NHE-3 and NHE-4 (Table 2) were tested. As expected, RT-PCR of rat kidney RNAs produced fragments of the proper size (~716 bp for NHE-3 and ~501 bp for NHE-4) but no amplifications for these isoforms were obtained in $RCCD_1$ cells. β-Actin was used as an internal control for kidneys and $RCCD_1$ cells in all experiments (Fig. 1*C*). These results demonstrate the presence of mRNAs for two different NHE isoforms in $RCCD_1$ cells. In addition, immunoblot experiments were performed using specific rat antibodies against NHE-1 and NHE-2. As shown in Fig. 1*D*, the NHE-1 antibody recognized two protein bands of the expected size [15] in both kidney and $RCCD_1$ cells (~96 and ~115 kDa). NHE-2 antibody recognized a major band of ~90 kDa in the rat kidney and in $RCCD_1$ cells, similar to that previously reported [6]. These results confirm the expression of NHE-1 and NHE-2 isoforms in $RCCD_1$ cells (Fig. 1*D*).

Basal Intracellular pH Regulation in $RCCD_1$ Cells

Intracellular pH measurements were performed in $RCCD_1$ monolayers grown on permeable filters, showing a basal pH_i of 7.44 ± 0.02 (n = 75). Experiments were performed using an HCO_3^--free medium (Solution A) to minimize bicarbonate-transporting systems. Figure 2 shows that basolateral Na^+ removal significantly reduced the basal pH_i by 0.08 ± 0.02 units (ΔpH_i, $P < 0.01$, $n = 6$), while apical Na^+ removal did not change basal pH_i. These results suggest that Na^+/H^+ exchanger is involved in pH_i regulation. So as to determine which isoform(s) may be involved in this process, the NHE-1-selective inhibitor HOE-694 was used. The pH_i was not modified by basolateral or apical addition of 1 μM HOE-694 (Fig. 2), a concentration predicted to produce almost complete inhibition of NHE-1 but not of NHE-2 [17, 18]. In contrast, basolateral but not apical addition of 10 μM HOE-694 significantly reduced the basal pH_i to a value similar to the one observed in absence of basolateral Na^+ (Fig. 2). These results suggest that basolateral

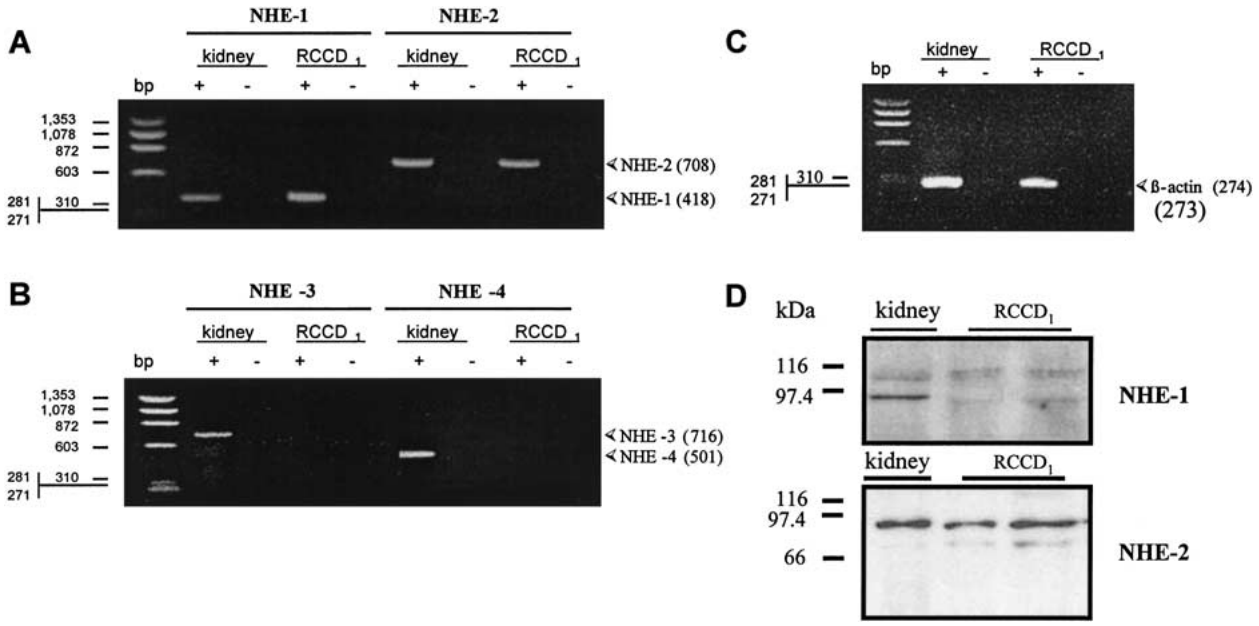


Fig. 1. NHE-isoform expression in $RCCD_1$ cells. (*A–C*) Representative RT-PCR experiments performed using specific primers for rat NHE isoforms in mRNAs from kidney (as positive control) and $RCCD_1$ cells. An aliquot (10 μl) of each reaction was subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. Assays were carried out in the presence (+) or absence (−) of RT enzyme. Left lane, ϕ X174 *Hae III* digested marker (bp). RT-PCR experiments using specific primers for: (*A*) rat NHE-1 and NHE-2 isoforms; (*B*) rat NHE-3 and NHE-4 isoforms; (*C*) β-actin, used as an internal control. (*D*) Western-blot studies using specific rat antibodies for NHE-1 and NHE-2 isoforms in kidney and $RCCD_1$ cells. The experiment shown is representative of at least four other assays.

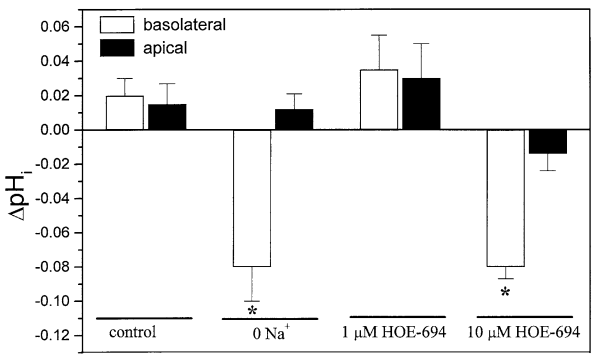


Fig. 2. NHE activity at steady-state pH_i. Intracellular pH was measured after an apical solution change (*black bars*) or basolateral solution change (*white bars*). ΔpH_i is the difference between initial pH_i (basal) and pH_i after the medium change either from the basolateral or from the apical bath. In control experiments, solution A was changed by solution A (sham); in $0Na^+$ experiments, solution A was changed by solution B; and in HOE-694 experiments, solution A was changed by solution A + 1 μM or 10 μM HOE-694. * $P < 0.01$ ($n = 6$), Student's *t*-test comparing experimental *vs.* sham experiments (control).

NHE-2 is the major isoform involved in steady-state pH_i regulation.

Intracellular pH Recovery from an Acid Load

To determine Na^+/H^+ exchange activity after acidification, $RCCD_1$ cells were acidified by the NH_4Cl prepulse technique [7]. Figure 3 summarizes the results, in which experiments were done after a 20-mM NH_4Cl pulse (Solution C), followed by removing apical and basolateral Na^+ (Solution B). Then, Na^+ was either restituted or not to apical, basolateral or both baths. As shown in Fig. 3*A* in absence of apical and basolateral Na^+ no pH_i recovery was observed. In contrast, sodium restitution to both sides (*control*) clearly induced pH_i recovery. Figure 3*B* shows that basolateral Na^+ removal reduced pH_i recovery markedly. In contrast, apical Na^+ removal did not affect pH_i recovery, but recovery was significantly lower in the presence of basolateral 100 μM EIPA, an inhibitor of Na^+/H^+ exchange (Fig. 3*C*). On the other hand, no sensitivity to apical EIPA was observed when Na^+ was removed only from the basolateral bath (Fig. 3*D*). The initial rate of pH_i recovery in all of these conditions are summarized in Fig. 4. These functional results indicate that pH_i regulation is totally dependent on the presence of Na^+ in the basolateral medium and sensitive to basolateral EIPA, suggesting basolateral Na^+/H^+ exchange activation after an acid load.

HOE-694 was used in order to evaluate which isoform may be involved in this response. The addition of 1 μM HOE-694 to basolateral membrane significantly inhibited pH_i recovery (ΔpH_i/min, control: 0.331 ± 0.06, $n = 5$ *vs.* 1 μM HOE-694: 0.125 ± 0.023, $P < 0.02$, $n = 5$). No additional inhibition was observed by 10 μM HOE-694 (ΔpH_i/min, 0.101 ±

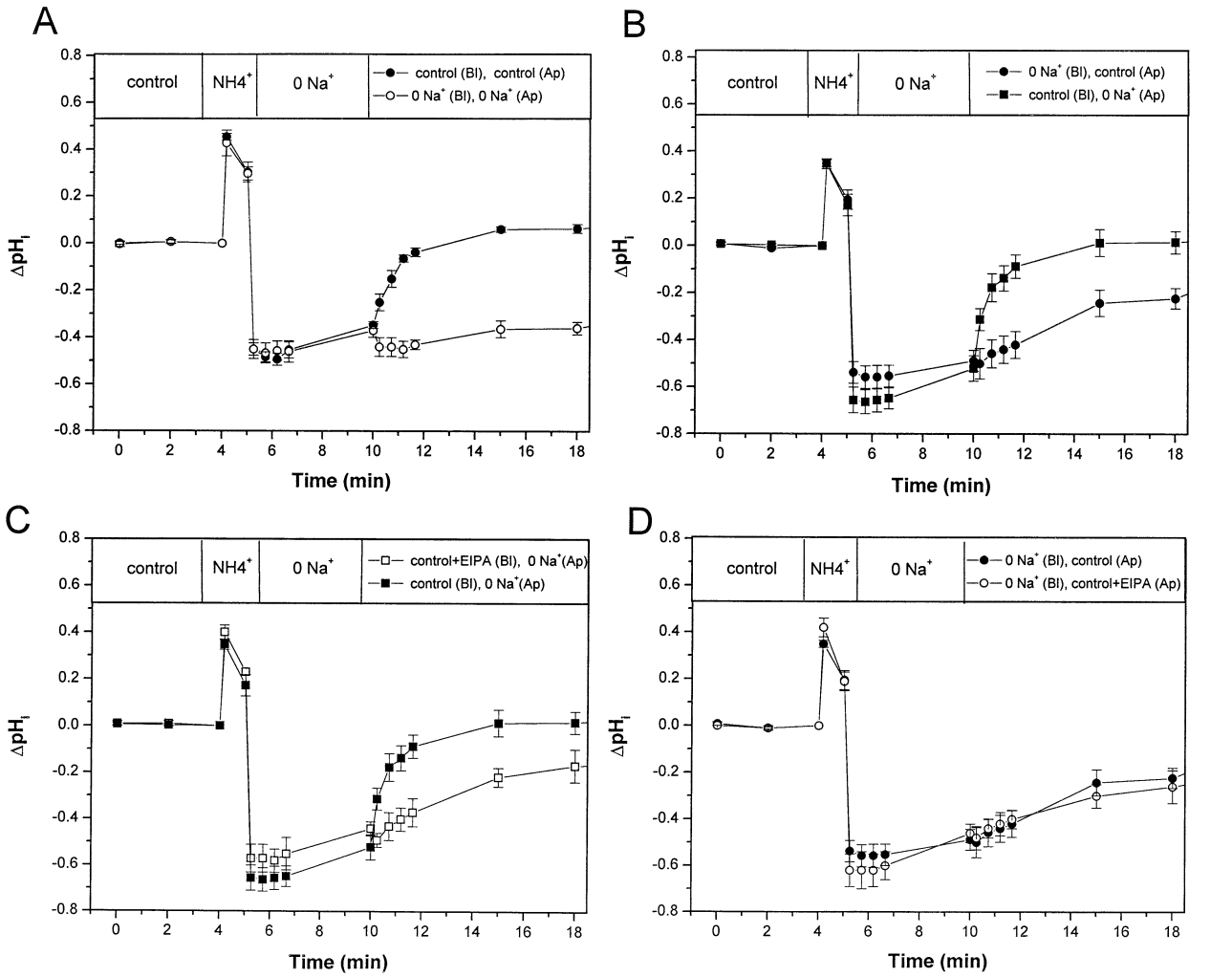
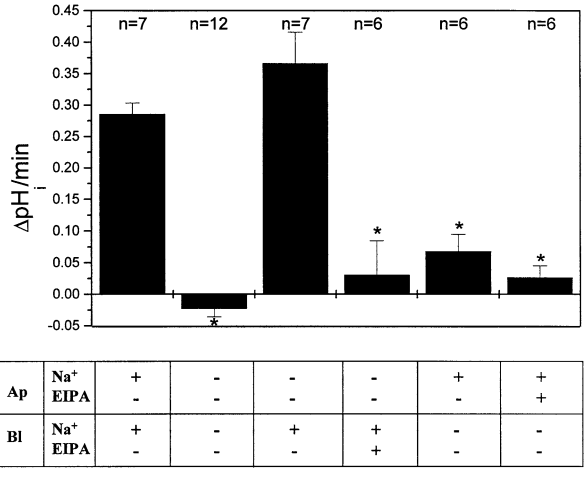


Fig. 3. Time course of pH_i regulation after an acid load. After 1 min incubation in NH_4Cl-containing solution (Sol. C), removal of NH_4Cl resulted in rapid cell acidification, which is maintained in absence of external Na^+. (*A*) Cellular acidification was recovered when Na^+ was restituted. (*B*) pH_i recovery was dependent on basolateral, but not apical, external Na^+. (*C*) Effect of basolateral 100 μM EIPA on pH_i recovery when Na^+ was removed from the apical bath. (*D*) Effect of apical 100 μM EIPA on pH_i recovery when Na^+ was removed from the basolateral bath. Values are the mean ± SE of 6–12 experiments.

0.010, $n = 5$, N.S), indicating that NHE-1 is the major isoform involved in pH_i regulation after an acid load.

Effects of Hyperosmolality on Basolateral Na^+/H^+ Exchange

It was previously reported that hyperosmolality activates Na^+/H^+ exchange in several systems [22, 33], however, no studies were performed in CCD cells. In this study, we evaluate whether the hyperosmolality affects pH_i in $RCCD_1$ cells. Cells incubated in buffer solution (Solution D, 300 mOsm/kgH_2O) were then exposed to a hypertonic solution either from the apical, the basolateral or both baths (Solution D plus 100 mM mannitol, 400 mOsm/kgH_2O). As shown in Fig. 5*A*, pH_i rapidly and significantly increased when osmotic challenge was performed from the basolateral bath (ΔpH_i/min: 0.130 ± 0.020, $n = 4$) or simultaneously from apical and basolateral bath (ΔpH_i/min: 0.137 ± 0.034, $n = 4$). Apical challenge only induced a slight alkalinization (ΔpH_i/min: 0.043 ± 0.020, $n = 4$). Hyperosmotic gradients were also generated with other osmolytes (sucrose and urea). As shown in Fig. 5*B*, hyperosmotic mannitol solution increased pH_i, reaching a maximal value at 3 min (ΔpH_i: 0.270 ± 0.051, $n = 4$). Hyperosmotic sucrose solution also increased pH_i, but this effect was smaller than that generated by mannitol (ΔpH_i: 0.15 ± 0.02 at 3 min, $n = 4$). In contrast, hyperosmotic urea solution did not induce cell alkalinization (ΔpH_i: 0.006 ± 0.026 at 3 min, $n = 4$). The more important response observed in the presence of a basolateral osmotic gradient generated by an impermeable solute



Ap	Na^+	+	-	-	-	+	+
	EIPA	-	-	-	-	-	+
Bl	Na^+	+	-	+	+	-	-
	EIPA	-	-	-	+	-	-

Fig. 4. Na^+-dependent pH_i recovery in $RCCD_1$ cells. Initial rate of pH_i recovery in absence of apical and/or basolateral Na^+ and effect of 100 μM EIPA. Values are expressed as ΔpH_i/min and compared to control solution (with Na^+). *$P < 0.001$, $n = 6$, Student's t-test for unpaired data.

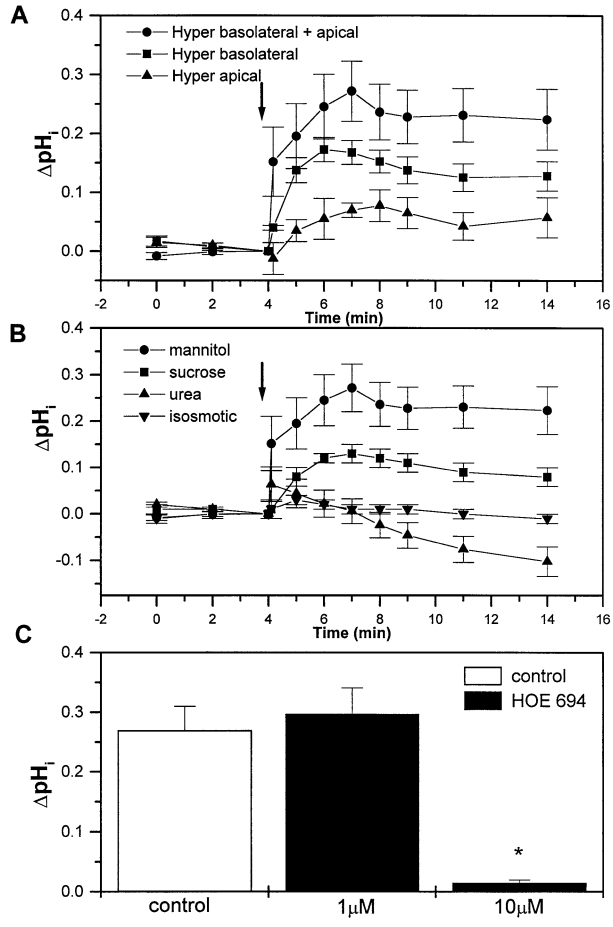


Fig. 5. Effects of hyperosmolality on NHE activity. (*A*) $RCCD_1$ cells were bathed with solution D; at arrow, 100 mOsM mannitol was added to apical and/or basolateral bath. It can be observed that basolateral, but not apical, hypertonicity caused significant cell alkalinization. (*B*) $RCCD_1$ cells were first exposed to solution D; at arrow, 100 mOsM mannitol, sucrose or urea was added to the bath. (*C*) ΔpH_i after 5 min incubation of $RCCD_1$ cells in 100 mOsM mannitol (control) or 100 mOsM mannitol plus HOE-694 (1 μM or 10 μM). A complete inhibition of the mannitol-induced alkalinization was observed at 10 μM of the drug, indicating NHE-2 activity. Values are the mean ± SE of 6–12 experiments. * $P < 0.001$, Student's t-test.

(mannitol) could be related to a differential membrane water osmotic permeability (basolateral higher than apical). To evaluate this possibility, water flux measurements were performed using the system previously described [20]. Our results show that water osmotic flux (J_w) was significantly higher when an osmotic gradient was performed from the basolateral bath (J_w μl.min^{-1}.cm$^{-2.}$ basolateral 0.537 ± 0.037 *vs.* apical 0.248 ± 0.009, $P < 0.001$, $n = 9$). These results would indicate that basolateral osmotic shock is more effective than apical one in producing cellular shrinkage and, consequently, cellular alkalinization. These events could also be supported by the basolateral presence of NHE isoform.

To evaluate whether NHE isoforms are involved in this alkalinization, $RCCD_1$ cells were treated with different concentrations of HOE-694. Exposure of cells to the hyperosmotic mannitol solution in the presence of 1 μM HOE-694 did not block pH_i alkalinization; in contrast, 10 μM HOE-694 significantly blocked pH_i increase (Fig. 5*C*). These findings indicate that hyperosmotic mannitol activates the NHE-2 isoform to cause cell alkalinization in $RCCD_1$ cells.

Discussion

We have previously demonstrated that $RCCD_1$ appears to be the first rat CCD cell line with high transepithelial resistance and properties of vectorial ion and water transport close to that of intact rat CCD. Moreover, this cell line expresses characteristics of two different cell types that may correspond to the principal and intercalated cells, present in the native cortical collecting ducts [4, 9, 19]. Therefore, it represented an attractive model to study the expression of NHE isoforms and their relative roles in CCD cells. Our present studies show the expression of NHE-1 and NHE-2 isoforms, but not NHE-3 or NHE-4, in $RCCD_1$ cells. Basal pH_i was around 7.4, similar to that previously reported for native rat CCD [32]. In absence of HCO_3^-, basolateral Na^+ removal significantly reduced the basal pH_i, suggesting Na^+/H^+ exchange activity. The availability of inhibitors with differences in affinity, such as HOE-694, allowed us to evaluate the relative contributions of the different NHE isoforms. Our results showed that basolateral (but not apical) addition of this drug

significantly reduced pH_i only at doses necessary to block the NHE-2 isoform. These findings strongly suggest that under basal conditions a basolateral NHE-2 isoform is involved in maintaining pH_i in $RCCD_1$ cells.

We have also demonstrated that after intracellular acidification by NH_4Cl, pH_i spontaneously and rapidly recovers (in the nominal absence of CO_2/HCO_3^-). This recovery was basolateral Na^+-dependent and sensitive to doses of HOE-694 necessary to completely block almost all NHE-1 activity [18]. Thus, these findings clearly indicate that the basolateral NHE-1 isoform is the primary mechanism for pH_i regulation after an intracellular acid load in $RCCD_1$ cells.

It has been previously proposed that hyperosmolality increases NHE-2 mRNA expression [33] and that transcriptional regulation of the NHE-2 gene is mediated by two osmotic response elements in mIMCD3 cells [1]. However, in CCD cells, it is not yet known whether hyperosmolality activates the Na^+/H^+ exchanger. In this study, we demonstrated that hyperosmotic mannitol and sucrose activate NHE to cause cell alkalinization, but that hyperosmotic urea causes no effect in $RCCD_1$ cells. Cell alkalinization induced by hyperosmotic shock was completely inhibited by pretreatment with 10 μM of basolaterally applied HOE-694. In contrast, 1 μM HOE-694 did not affect pH_i. Therefore, these findings propose that NHE-2 is involved in intracellular alkalinization after a hyperosmotic treatment. Since mannitol and sucrose are relatively impermeant solutes, it is most likely that they make the solution hypertonic. In contrast, urea is freely permeant and raises the osmolality, but does not affect the solution tonicity. Therefore solution tonicity, rather than absolute osmolality, seems to be the important factor for the NHE-2 activation in CCD cells. We have also shown that basolateral hypertonic mannitol solution induced a cell alkalinization together with a higher osmotic water flux when compared with apical hypertonic mannitol solution. Therefore, CCD cells show rectification, i.e., the same osmotic gradient produces different fluid flows when the direction of the gradient is reversed. Epithelia rectification was previously demonstrated in toad bladder [8]. Regardless of the mechanism, basolateral hyperosmotic shock may cause more cellular shrinkage and consequently more activation of NHE. This rapid activation of NHE-2 would be crucial for cell volume regulation in CCD cells.

From our functional studies, we propose that in $RCCD_1$ cells, NHE-1 and NHE-2 are both expressed in the basolateral membrane, but that they have different roles. Basolateral localization of NHE-1 confirms previous reports in CCD cells and in IMCD cells [3, 27, 34]. The basolateral localization of NHE-2 is in agreement with published reports in IMCD3 tubule [34] and in a mouse inner medullary collecting duct cell line (mIMCD-3) [33]. However, no studies about NHE-2 localization were performed in native CCD. Only one study proposes apical localization of NHE-2 in a rabbit cortical collecting duct cell line [23]. In summary, most of the studies performed in the collecting duct tubules showed that NHE-2 is limited to the basolateral membrane, contrary to that observed in other nephron segments [15, 35]. It is tempting to speculate that the discrepancies about NHE-2 localization may be related to the existence of splice variants or posttranslational truncation of NHE-2, as was previously proposed [16]. Another possible explanation to understand the differences in NHE-2 localization may be due to the fact that the half-life of plasma membrane NHE-2 is relatively short (3 hr), compared with that of NHE-1 and NHE-3, as was reported for fibroblast (PS120) and epithelial (Caco-2) cells [11]. This rapid turnover could be important in maintaining basal levels of NHE-2 and also for altering transporter levels in the regulated state. Taking in account these findings, we can speculate that NHE-2 could, in part, be located intracellularly, as previously shown in other systems [10, 38], Cavet et al. demonstrated that "basal" NHE-2 transport is not affected by the PI3-kinase in fibroblasts [11]. However, it remains to be determined whether trafficking of a subpopulation of intracellular NHE-2 plays a role in the "regulated state" in either PI3 kinase-dependent or -independent manner [11]. Moreover, apical or basolateral targeting could be related to the existence of plasticity in NHE-2 expression. Significant plasticity of cortical collecting cell function, reflected by changes in the activity of apical and basolateral acid-base transporters (Cl^-/HCO_3^-, H^+-pumps), has been observed and shown to be related to the acid-base states [28].

In conclusion, our findings let us propose that in $RCCD_1$, a cell line that expresses characteristics of native CCD cells, NHE-1 is involved in pH_i regulation after intracellular acidification. In contrast, NHE-2 is mainly involved in maintaining steady-state cellular pH_i and cell volume regulation. Therefore, the $RCCD_1$ cell line is a useful model to further investigate the volume regulatory mechanisms for the maintenance of cell volume in mammalian cortical collecting duct cells.

We thank Dr. Maurice Bichara for helpful comments on the manuscript. We are grateful to Dr. Juergen Puenter (Aventis Pharma Deutschland GmbH, Germany) who kindly provided us with the HOE-694 compound. This work was supported by grants from Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET, Argentina); Universidad de Buenos Aires, Argentina; Fundación ANTORCHAS. (Argentina) and Ministerio de Salud de la Nación Argentina (Becas Ramón Carrillo–Arturo Oñativia, Argentina).

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