

Distinct Structural Domains Confer cAMP Sensitivity and ATP Dependence to the Na⁺/H⁺ Exchanger NHE3 Isoform*

(Received for publication, September 20, 1995, and in revised form, December 5, 1995)

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Agents known to increase cAMP levels in renal and intestinal epithelia decrease sodium absorption by inhibiting NHE3, an isoform of the Na⁺/H⁺ exchanger expressed at high levels in apical membranes of these cells. In contrast, the ubiquitous, housekeeping isoform of the exchanger (NHE1) is stimulated by cAMP in some cell types. Optimal activity of NHE3 as well as NHE1 requires the presence of ATP. To gain insight into the molecular mechanisms of ATP dependence and cAMP regulation of NHE3, a series of mutations were constructed by progressively truncating segments of the C-terminal cytoplasmic domain of the transporter at amino acid positions 684, 638, and 579 (named NHE3Δ684, NHE3Δ638, and NHE3Δ579). In addition, chimeric antiporters were constructed with the N-terminal transmembrane domain of NHE3 linked to the entire cytoplasmic region of NHE1 (chimera NHE3/1) or *vice versa* (chimera NHE1/3). These constructs were heterologously expressed in antiport-deficient Chinese hamster ovary cells, and their activities were assessed by fluorimetric measurements of intracellular pH and by radioisotope determinations of Na⁺ influx. Forskolin, which directly stimulates adenylate cyclase, inhibited NHE3 as well as NHE1/3, but not NHE3/1, suggesting that the cytoplasmic domain of NHE3 was sufficient to confer sensitivity to inhibition by cAMP. Forskolin also inhibited the truncated mutant NHE3Δ684 to an extent similar to that for wild type NHE3. However, the inhibitory effect was greatly reduced in NHE3Δ638 and more profound truncations (NHE3Δ579) obliterated the effect of forskolin. These findings suggest that a region found between amino acids 579 and 684 is essential for the cAMP response of NHE3. In contrast, comparable ATP dependence was observed in all exchanger constructs examined. These observations indicate that ATP dependence is conferred by a region of the molecule in or adjacent to the transmembrane domain, which is most conserved between isoforms. It is concluded that different sites, and therefore different mechanisms, underlie inhibition of NHE3 by cAMP and by depletion of ATP.

Na⁺/H⁺ exchange (NHE)¹ activity is present in virtually all mammalian cells and catalyzes the electroneutral exchange of one sodium for one proton. The exchange of Na⁺ for H⁺, which is characteristically sensitive to inhibition by amiloride and its analogs (1, 2), is driven by the concentration gradients of these ions and does not require direct expenditure of metabolic energy. Nevertheless, the presence of ATP is required for optimal exchanger activity by a mechanism that is poorly understood (3).

NHE activity is thought to be essential for pH homeostasis (4, 5), transepithelial ion and water transport (6), and cell volume regulation (7) and may also play a role in cell proliferation (5) and adhesion (8, 9). This functional versatility prompted the search for variant forms of the transporter. To date, five mammalian isoforms of the Na⁺/H⁺ exchanger (NHE1 to NHE5) have been identified (10–15). The isoforms share a similar hydropathy profile that indicates the existence of two major structural domains: a predominantly hydrophobic transmembranous N terminus and a more hydrophilic cytoplasmic C terminus. The N-terminal domain, which is highly conserved among isoforms, is predicted to span the membrane 10–12 times and is believed responsible for catalyzing Na⁺ and H⁺ exchange and conferring amiloride sensitivity (16, 17). The more variable C terminus is thought to extend into the cytosol and to play a role in regulating the activity and subcellular distribution of the exchangers (16). It contains potential sites for phosphorylation by protein kinases and, in some isoforms, includes a segment with affinity for calmodulin (10, 11, 18–20).

NHE1, the “housekeeping” isoform, is present in nearly all mammalian cells examined to date. In epithelial cells, it localizes predominantly to the basolateral membrane. The other isoforms have a more restricted tissue distribution. NHE2 to NHE4 are abundant in epithelial cells of kidney, intestine, and stomach, whereas NHE5 resides primarily in brain, spleen, and testis. Of these, NHE3 has been studied in most detail. It is restricted to the apical (brush border) membranes of some epithelial cells of the renal and gastrointestinal tracts, where it mediates Na⁺ reabsorption (21–23). In these tissues, the rate of Na⁺ reabsorption is variable and stringently controlled, primarily by agents that modulate the levels of adenosine 3',5'-cyclic monophosphate (cAMP). Because NHE3 is the predominant exchanger in epithelial brush border membranes, this isoform is postulated to be the direct target of the cAMP-dependent protein kinase A (PKA) (24). However, direct demonstration of the regulation of NHE3 by PKA in native tissues is

* This work was supported in part by the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Supported by a research scholarship from the Fonds de la Recherche en Sante du Quebec.

¹ The abbreviations used are: NHE, Na⁺/H⁺ exchanger; BCECF, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; [H⁺]_i, intracellular proton concentration; HOE 694, (3-methylsulfonyl-4-piperidinobenzoyl)guanidine methanesulfonate; pH_i, cytosolic pH; pH_o, extracellular pH; PKA, adenosine 3',5'-cyclic monophosphate-dependent protein kinase A.

complicated by the coexistence of multiple isoforms in different cell types and even in different membranes of the same cell. These confounding factors can be overcome by heterologous transfection of NHE3 into cells devoid of endogenous exchanger activity. Using this approach, it has recently been demonstrated that the activity of NHE3 is markedly depressed by elevating intracellular cAMP using agents such as forskolin.²

The molecular basis of the regulatory effects of cAMP on NHE3 is not clear. The cytosolic domain of this isoform contains several consensus sites for phosphorylation by PKA (11), some of which may mediate the observed inhibition. On the other hand, phosphorylation-independent regulation of other isoforms has been described (7, 25). In an attempt to better understand the regulation of NHE3, a mutational analysis was conducted by generating a series of progressive truncations of the regulatory C-terminal domain that contains the putative phosphorylation sites. For comparative purposes, the behavior of NHE1 was also examined since, in osteoblasts, it has been reported to be *activated* by cAMP (26). Definition of the site(s) that confer NHE3-specific sensitivity to cAMP was accomplished by creating chimeras of NHE1 and NHE3. The functional behavior of these mutants was studied in isolation by transfection into AP-1 cells, a Chinese hamster ovary line that lacks endogenous Na^+/H^+ exchange (27). These transfectants were also used to analyze the structural basis of the paradoxical ATP dependence of the exchanger.

EXPERIMENTAL PROCEDURES

Materials, Solutions, and Antibodies—Nigericin and 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) acetoxymethyl ester were from Molecular Probes, Inc. Antimycin A, deoxyglucose, pepstatin A, phenylmethylsulfonyl fluoride, iodoacetamide, forskolin and amiloride were obtained from Sigma. The BCA protein assay reagent was purchased from Pierce. HOE 694 ((3-methylsulfonyl-4-piperidinobenzoyl)-guanidine methanesulfonate) was a generous gift from Hoechst AG. Enhanced chemiluminescence reagents were from Amersham Corp.

Polyclonal antibodies to the NHE1 isoform of the Na^+/H^+ exchanger were raised by injecting rabbits with a fusion protein constructed with β -galactosidase of *Escherichia coli*, containing the last 157 residues (658–815) of the human NHE1. Polyclonal antibodies to the NHE3 isoform were generated against a glutathione S-transferase fusion protein with residues 565–690 of the rat NHE3. Antibodies were affinity-purified as described (7, 28).

Bicarbonate-free medium RPMI 1640 was buffered with 25 mM HEPES to pH 7.3 and adjusted to 290 ± 5 mosM. Phosphate-buffered saline consisted of (in mM): 140 NaCl, 10 KCl, 8 sodium phosphate, 2 potassium phosphate, pH 7.4. The isotonic Na^+ -rich medium used in the fluorimetric pH measurements contained (in mM): 140 NaCl, 3 KCl, 1 MgCl_2 , 10 glucose, 20 HEPES, pH 7.3. The isotonic Na^+ -free medium contained the same salts, but NaCl was substituted by *N*-methyl-D-glucamine. The isotonic K^+ -rich medium had the same composition as Na^+ -rich medium, except that NaCl was replaced by KCl. In all cases the osmolarity was set to 290 ± 5 mosM with the major salt.

Construction of Na^+/H^+ Exchanger Chimeras and Deletion Mutations—Complementary DNA fragments of rat NHE1 (NotI–NsiI fragment, nucleotides –761 to +3820) and NHE3 (KpnI–SmaI fragment, nucleotides –44 to +4288) were initially subcloned into the mammalian expression plasmid pCMV and called pCMV/NHE1 and pCMV/NHE3, respectively, as described previously (12). To facilitate construction of Na^+/H^+ exchanger chimeras and deletion mutations, it was necessary to remove parts of the 5'- and/or 3'-untranslated regions of NHE1 and NHE3 in order to create a number of useful restriction endonuclease sites. For NHE1, nucleotides –761 to –42 in the 5'-untranslated region were removed by PCR mutagenesis. As well, nucleotides +3454 to +3820 in the 3'-untranslated region and part of the pCMV polylinker region were eliminated by removing an XbaI–XbaI fragment. For NHE3, nucleotides +2950 to +4288 in the 3'-untranslated region and part of the polylinker were eliminated by removing an NsiI–NsiI fragment. A unique EagI restriction endonuclease site was then engineered into each cDNA by site-directed mutagenesis (29) at the predicted

junction between the N-terminal transmembranous and the C-terminal cytoplasmic regions. For NHE1, the introduction of an EagI site (CGG CCG) at amino acid positions 504–505 did not alter its coding sequence (i.e. Arg-Pro). For NHE3, insertion of an EagI site at amino acid positions 454–455 resulted in a conservative substitution, altering its coding sequence from Lys-Pro to Arg-Pro. The cDNAs were sequenced to confirm the presence of the EagI site and to ensure that other random mutations were not introduced during the mutagenesis procedure. These modified plasmids were called pNHE1 and pNHE3, respectively (Fig. 1A). Chimeric Na^+/H^+ exchangers were constructed by the reciprocal exchange of the EagI–XbaI fragments, which comprised the entire C-terminal cytoplasmic regions of NHE1 and NHE3 to generate NHE1/3 and NHE3/1 (Fig. 1B). Mutations of NHE3 were also constructed by deleting segments of the C-terminal cytoplasmic region according to the following procedures (Fig. 1B). NHE3 Δ 684 was generated by cleaving pNHE3 with BssHII and removing the BssHII–BssHII fragment of NHE3. The resulting linear plasmid containing the BssHII 5' overhang ends was then blunt-ended by a fill-in reaction using Klenow, followed by cleavage of a single downstream ApaI site in the polylinker to remove the intervening fragment. The ApaI 3' overhang end was subsequently blunt-ended by the 3' exonuclease activity of T4 DNA polymerase, and the remaining linear plasmid was then religated with T4 DNA ligase. This procedure resulted in NHE3 being truncated at amino acid position 684, followed by the addition of two non-native amino acids (Ile-Leu) and a TAG stop codon in the proper reading frame. NHE3 Δ 638 was generated by cleaving pNHE3 with SacI and removing a SacI–SacI fragment containing the entire coding region up to amino acid position 638. This SacI–SacI fragment was subcloned into a modified pCMV vector where the bulk of the multiple cloning site was removed (BamHI to XbaI) in order to leave a unique SacI site. Plasmids containing the SacI–SacI fragment in the proper orientation relative to the cytomegalovirus promoter were selected for further analysis. This procedure resulted in NHE3 being truncated at amino acid position 638 followed by the addition of two non-native amino acids (Gly-Ser) and a TAG stop codon in the reading frame. NHE3 Δ 579 was generated by engineering a KasI site in pNHE3 at amino acid positions 578–579. The modified pNHE3 was then cleaved with KasI and XbaI to remove the 3' terminal KasI–XbaI fragment, which contained amino acids 580–831. The remaining linear plasmid containing 5' overhang ends were blunt-ended by a fill-in reaction using Klenow and then religated with T4 DNA ligase. This procedure resulted in an in-frame TAG stop codon being placed immediately adjacent to amino acid position 579 and the penultimate amino acid being changed from Glu to Gly. The KasI site was not present in the other cDNA constructs. All deletion mutations were sequenced at the C-terminal ends to verify the constructions.

Stable Transfection and Expression of the Na^+/H^+ Exchanger Chimeric and Deletion Mutation cDNAs—Chemically mutagenized Chinese hamster ovary (AP-1) cells devoid of endogenous Na^+/H^+ exchange activity (27) were transfected with plasmids containing the various Na^+/H^+ exchanger constructs by the calcium phosphate-DNA coprecipitation technique of Chen and Okayama (30). Starting 48 h after transfection, the AP-1 cells were selected for survival in response to repeated (5–6 times over a 2-week period) acute NH_4Cl -induced acid loads (12, 31) in order to discriminate between Na^+/H^+ exchanger positive and negative transfectants.

Cell Culture—Transfected cells were grown in a humidified 95% air, 5% CO_2 atmosphere and maintained in α -minimal essential medium supplemented with 10% fetal calf serum and antibiotic (penicillin/streptomycin). Cells expressing Na^+/H^+ exchanger activity were regularly selected by an acute acid challenge, as described above. This process was repeated to eliminate possible revertants and maintain stable functional expression of the exchanger.

ATP Depletion—For depletion of ATP, cells were incubated for 10 min in a glucose-free medium containing 100 mM potassium glutamate, 30 mM KCl, 10 mM NaCl, 1 mM MgCl_2 , 10 mM HEPES (pH 7.2), 5 mM deoxyglucose, plus 1 $\mu\text{g}/\text{ml}$ antimycin A as described (32). This high K^+ , low Na^+ , and Ca^{2+} -free medium was used to prevent Na^+ and/or Ca^{2+} loading of the cells upon inhibition of the Na^+/K^+ or Ca^{2+} pumps. Substitution of most of the Cl^- by the poorly permeant glutamate[–] minimizes cell swelling. This procedure reduces ATP content by >90%. Control cells were incubated for the same time in potassium glutamate medium devoid of deoxyglucose and antimycin A, but containing instead 10 mM glucose.

Measurement of Na^+/H^+ Exchanger Activity: Cytosolic pH Measurements—Stably transfected cells were grown to 60–70% confluence on glass coverslips and serum-starved for at least 16 h. Next, cells were incubated for 20 min in HPMEI or in potassium glutamate medium (to study the effect of forskolin or ATP depletion, respectively), with 2

² Kandasamy, R. Yu, F. H., Harris, R., Boucher, A., Hanrahan, J. W., and Orlowski, J. (1995) *J. Biol. Chem.* **270**, 29209–29216

$\mu\text{g/ml}$ BCECF acetoxymethylester at 37°C . Cells were pulsed with 50 mM NH_4Cl during the last 15 min of the incubation with BCECF and stimulation with forskolin or ATP depletion were performed during the last 10 min of incubation with the dye. Cells were next washed three times with NH_4Cl - and Na^+ -free solution and resuspended in this medium. In order to study the sodium-dependent pH_i recovery, cells were next incubated in isotonic Na^+ -rich medium. To measure the fluorescence of BCECF, the coverslip was placed in a thermostatted Leiden holder on the stage of a Nikon TMD-Diaphot microscope equipped with a Nikon Fluor $40\times$ oil-immersion objective. An Empix Imaging filter wheel was used to alternately position the two excitation filters (500 ± 10 and $440 \pm 10\text{ nm}$) in front of a xenon lamp. In order to minimize dye bleaching and photodynamic damage, neutral density filters were used to reduce the intensity of the excitation light reaching the cells. The excitation light was directed to the cells via a 510-nm dichroic mirror and fluorescence emission was collected by a $535 \pm 25\text{-nm}$ band-pass filter. Data were recorded every 10 s by irradiating the cells for 500 ms at each of the excitation wavelengths. The fluorescence image was captured on a Star 1 liquid-cooled CCD camera (Photometrics Ltd.), then downloaded to a Dell 486 computer where the Metafluor Imaging System software from Universal Imaging Corp. was used to acquire and ratio the two images created by dual excitation.

Calibration of the fluorescence ratio *versus* pH was performed for each experiment by equilibrating the cells in isotonic K^+ -rich medium buffered to varying pH values (between 7.45 and 5.85) in the presence of the K^+/H^+ ionophore nigericin ($5\text{ }\mu\text{M}$). Calibration curves were constructed by plotting the extracellular pH , which is assumed to be identical to the internal pH , against the corresponding fluorescence ratio (33).

Cellular buffering power was determined in the pH_i range studied by pulsing cells with weak electrolytes, as described (34). H^+ efflux rates (mm/s) were calculated by multiplying the rate of pH_i recovery (dpH_i/dt) times the buffering capacity, at the corresponding pH values. Na^+/H^+ exchange rates were analyzed using the general allosteric model described by the Hill equation shown in Equation 1.

$$V/(V_{\max} - V) = K[S]^n \quad (\text{Eq. 1})$$

or

$$V = (V_{\max} \cdot [S]^n)/(K^{-1} + [S]^n)$$

V is the H^+ (equivalent) efflux at a particular cytosolic H^+ concentration $[S]$; V_{\max} is the maximal H^+ efflux rate, and n is the Hill coefficient, a minimal estimate of the number of binding sites for H^+ . Though n was greater than 1, the data were adequately fit using a single intrinsic binding constant, K , suggesting that the affinities of the individual sites were of the same order. Experimental results were fit to the above model using a nonlinear regression data analysis program, Enzfitter from Bios Corp.

Measurement of $^{22}\text{Na}^+$ Influx— Na^+/H^+ exchanger activity was also measured as the rate of amiloride-inhibitable $^{22}\text{Na}^+$ influx at a constant intracellular H^+ concentration. For these studies, pH_i was set at 6.3 by incubating confluent cell monolayers on 24-well plates in medium containing the appropriate K^+ concentration and the K^+/H^+ exchange ionophore nigericin (33). Since at equilibrium $[\text{K}^+]_i/[\text{K}^+]_o = [\text{H}^+]_i/[\text{H}^+]_o$, the desired pH_i was calculated from the imposed $[\text{K}^+]$ gradient and the extracellular pH ($\text{pH}_o = 7.4$), assuming an intracellular $[\text{K}^+]$ of 140 mM . To clamp pH_i at 6.3, the cell monolayers were first washed with a N -methyl-D-glucamine $^+$ -balanced salt solution and then preincubated in a K^+ -nigericin solution containing 153 mM N -methyl-D-glucamine chloride, 11.2 mM KCl , 2 mM NaCl , 1 mM MgCl_2 , $10\text{ }\mu\text{M}$ nigericin, 10 mM HEPES, pH_o 7.4, for 3 min at room temperature. This solution was then removed and $^{22}\text{Na}^+$ influx measurements were initiated in the same K^+ -nigericin solution supplemented with $1\text{ }\mu\text{Ci}$ of $^{22}\text{NaCl}/\text{ml}$ and 1 mM ouabain (to inhibit Na^+/K^+ -ATPase and minimize $^{22}\text{Na}^+$ efflux) in the absence or presence of 2 mM amiloride. $^{22}\text{Na}^+$ influx was terminated after 10 min by aspirating the radiolabeled medium and rapidly washing the cells three times with ice-cold NaCl stop solution (130 mM NaCl , 1 mM MgCl_2 , 2 mM CaCl_2 , 20 mM HEPES- NaOH , pH 7.4). To extract the radiolabel, 0.25 ml of 0.5 N NaOH was added to each well and the wells were washed with 0.25 ml of 0.5 N HCl . Both the solubilized cell extract and wash solutions were suspended in 5 ml of scintillation fluid and the radioactivity assayed by liquid scintillation spectroscopy. $^{22}\text{Na}^+$ influx was linear with time for at least 10 min under these experimental conditions.

Membrane Isolation—Cells were grown to 80–90% confluence on 10-cm dishes and washed three times with ice-cold phosphate-buffered saline. Next, cells were scraped into a hypotonic solution containing 10 mM HEPES, 18 mM potassium acetate, 1 mM EDTA, $1\text{ }\mu\text{M}$ pepstatin, 1

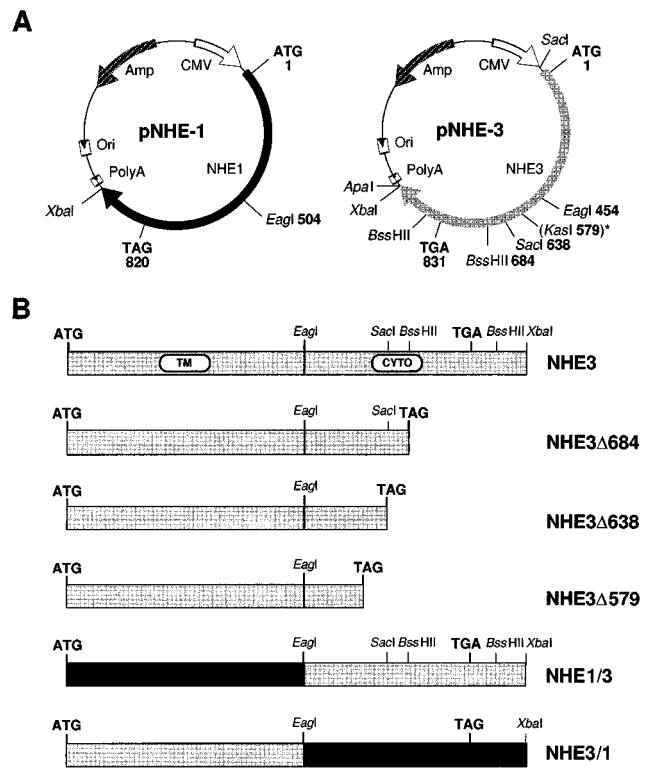


FIG. 1. Diagram of Na^+/H^+ exchanger chimeras and deletion mutants. A, diagram of the mammalian expression plasmids containing the rat parental Na^+/H^+ exchanger isoforms NHE1 and NHE3. Indicated in the figure are relevant restriction endonuclease sites used in the construction of chimeras of NHE1 and NHE3 and deletion mutations of NHE3. B, linear profile and nomenclature of chimeras of NHE1 and NHE3 and deletion mutations of NHE3. Details of their construction are provided under "Experimental Procedures."

mM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide (pH 7.2) and homogenized by 50 strokes of a Dounce homogenizer. The lysate was centrifuged at $1000 \times g$ for 5 min to remove nuclei and incompletely broken cells, and the resulting supernatant was sedimented at $100,000 \times g$ for 60 min at 4°C . The resulting microsomal pellet was resuspended in Laemmli sample buffer for electrophoresis.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—To assess the expression of the gene products, immunoblotting was carried out using polyclonal antibodies against NHE1 and NHE3. Samples containing $25\text{ }\mu\text{g}$ of membrane protein were subjected to electrophoresis in 7.5% acrylamide gels and transferred to nitrocellulose. Blots were blocked with 0.2% gelatin and exposed to one of the primary polyclonal anti-NHE antibodies described above. Affinity-purified antibodies against human NHE1 (residues 658–815) and NHE3 (residues 565–690) were used at 1:5000 dilution. The secondary antibody, goat anti-rabbit coupled to horseradish peroxidase, was applied at 1:5000 dilution. Immunoreactive bands were visualized using enhanced chemiluminescence (Amersham Corp.).

RESULTS

Structural Analysis of the Regulation of NHE3 by cAMP—A series of truncated mutants and chimeras was used to define the structural basis of the inhibitory effect of cAMP on the activity of NHE3. These are diagrammatically illustrated in Fig. 1. The mammalian expression plasmids containing the parental rat Na^+/H^+ exchanger isoforms NHE1 and NHE3 are shown in panel A. The diagram includes the relevant restriction endonuclease sites used in the construction of the deletion mutants of NHE3 and NHE1/NHE3 chimeras. Panel B of Fig. 1 illustrates the linear profile and nomenclature used to designate the resulting truncations and chimeras.

Na^+/H^+ exchange was initially assessed fluorimetrically in cells treated with or without forskolin, an agent that stimulates adenylate cyclase and increases intracellular cAMP. No at-

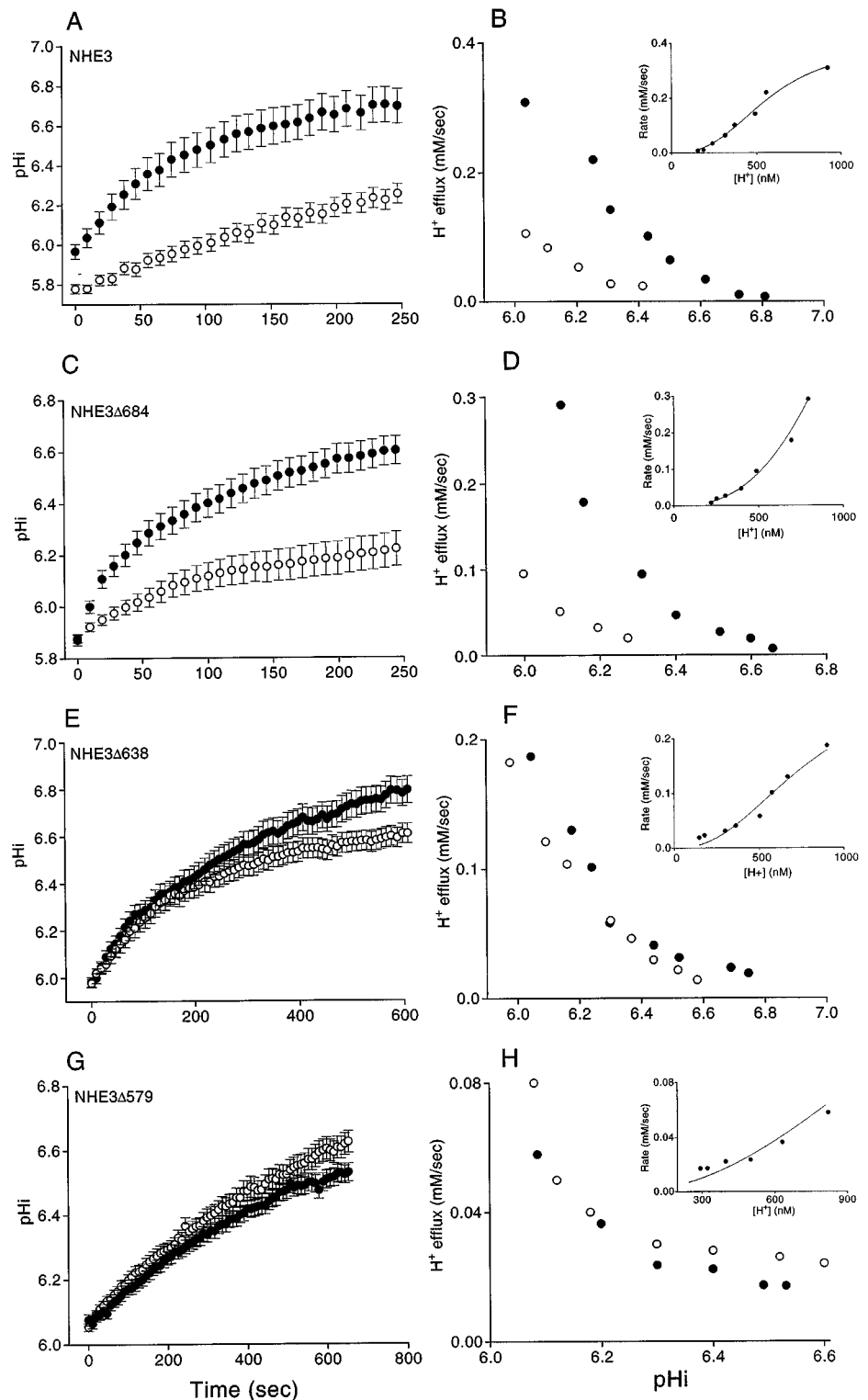


FIG. 2. Effect of forskolin on the activity of full-length and C-terminally truncated NHE3: pH determinations. AP-1 cells transfected with full-length NHE3 (A and B), NHE3 Δ 684 (C and D), NHE3 Δ 638 (E and F) or NHE3 Δ 579 (G and H) were loaded with BCECF in bicarbonate-free medium. The cells were acid-loaded with ammonium and treated with (open symbols) or without (solid symbols) 10 μ M forskolin during the final 10 min of incubation with the dye. Next, the pH_i recovery induced by addition of Na^+ was measured fluorimetrically. Results in each panel are the mean \pm S.E. of at least 20 cells from three separate experiments. Left panels illustrate the recovery of pH_i , while the right panels show the pH_i dependence of the rate of H^+ efflux, calculated from the mean $\Delta pH/\Delta t$ and the buffering power, as described under "Experimental Procedures." The insets show the rate as a function of $[H^+]$. Data points were determined experimentally, while the line was fitted using the Hill equation (see "Experimental Procedures").

tempt was made to compare the absolute rates of transport between stable transfectants, which can reflect parameters other than the length of the construct, such as the site(s) of genomic integration, the number of incorporated cDNA copies/cell, and differences in intracellular processing of mutated proteins. Acid-loaded cells were exposed to Na^+ , and the rate of recovery of pH_i was used as a measure of exchanger activity. It is noteworthy that in all cases the rate of alkalization was negligible in the absence of Na^+ . As shown in Fig. 2A, untreated cells transfected with full-length NHE3 recovered readily from the acid load upon reintroduction of Na^+ (solid

circles). Consistent with observations in renal proximal tubule cells (35, 36), pretreatment with forskolin markedly depressed the activity of NHE3 (open circles). To more precisely evaluate the mechanism of inhibition, the rate of Na^+/H^+ exchange was estimated at varying pH_i . H^+ (equivalent) fluxes were calculated from the rate of change of pH_i ($\Delta pH/\Delta t$) and the buffering power, determined independently throughout the pH range studied. As summarized in Fig. 2B, the H^+ efflux rate in control cells was highest at acidic pH_i and decreased thereafter approaching quiescence near pH 6.8. As described in isolated renal brush borders (1), the exchange process showed cooper-

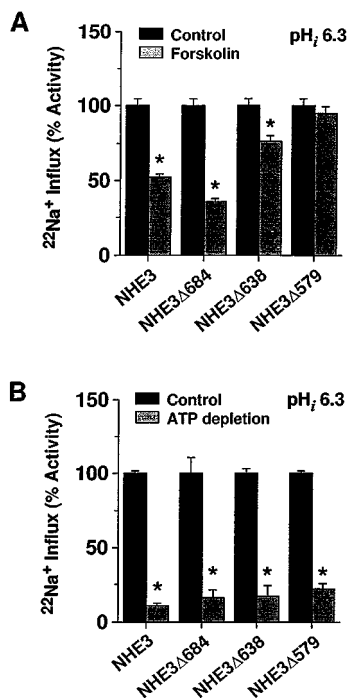


FIG. 3. Effects of forskolin and ATP depletion on the activity of full-length and C-terminally truncated NHE3: Na^+ uptake determinations. AP-1 cells transfected with either full-length NHE3, NHE3 Δ 684, NHE3 Δ 638 or NHE3 Δ 579 were grown to confluence on 24-well plates. **A**, the monolayers were washed twice with Na^+ -medium (see "Experimental Procedures" for composition of media). The cells were then preincubated in the same solution in the absence or presence of 10 μM forskolin for 15 min at 37 $^{\circ}\text{C}$. Next, the medium was removed and the cells were preincubated for 3 min in K^+ -nigericin solution to clamp the intracellular pH at 6.3 in the continued absence or presence of 10 μM forskolin. At the end of this equilibration period, the solutions were aspirated and replaced with the same media supplemented with $^{22}\text{NaCl}$ (1 $\mu\text{Ci}/\text{ml}$), 1 mM ouabain, with or without 2 mM amiloride. Radioisotope uptake was terminated after 10 min by aspiration and extensive washing with ice-cold Na^+ -solution and the samples were processed as described under "Experimental Procedures." Background $^{22}\text{Na}^+$ influx that was not inhibitable by 2 mM amiloride was subtracted from the total influx. Data are expressed as the mean percentage of amiloride-inhibitable $^{22}\text{Na}^+$ influx \pm S.E. ($n = 16$) from four independent experiments. **B**, the cell monolayers were incubated for 10 min in control or ATP-depleting solutions at 37 $^{\circ}\text{C}$. Na^+/H^+ exchanger activity was then measured as described above. Data are expressed as the mean percentage of amiloride-inhibitable $^{22}\text{Na}^+$ influx \pm S.E. ($n = 8-12$) from 2-3 independent experiments. Significance of differences between control and experimental measurements was calculated using two-tailed Student's t test and is indicated by an asterisk ($p < 0.001$).

activity with respect to H^+ . The data were fit adequately by the Hill equation (Fig. 2*B*, *inset*), yielding a coefficient of ≈ 3 . Treatment with 10 μM forskolin reduced Na^+/H^+ exchange activity at all pH_i values tested. Importantly, the pH_i dependence of the exchanger was shifted to more acidic levels by nearly 0.4 units. The Hill coefficient was not markedly different when cAMP was elevated (≈ 2.7), suggesting that cooperativity with respect to H^+ persisted under these conditions. Therefore, the effect of cAMP appears to be largely due to a reduced affinity for intracellular H^+ . The effect of cAMP on V_{max} could not be properly assessed due to insufficient data points below pH 6.0. Such data were not collected because of concerns regarding potential cellular damage caused by extremely acidic conditions. In this figure, the data are means \pm S.E. of 27/54 (control/treated) individual determinations.

The inhibitory effect of forskolin on NHE3 was confirmed measuring amiloride-sensitive Na^+ uptake. As illustrated in Fig. 3*A*, activation of the adenylate cyclase reduced influx by nearly 50% at pH_i 6.3, in good agreement with the fluorescence

determinations of pH_i .

The behavior of the Δ 684 truncation was almost indistinguishable from that of the full-length NHE3. As shown in Fig. 2 (*C* and *D*), this mutant displayed similar recovery rates, a sharp pH_i dependence and cooperativity with a Hill coefficient of ≈ 2.6 . Importantly, NHE3 Δ 684 was also susceptible to inhibition by cAMP which, as in wild type NHE3, was manifested as an acidic shift in the pH_i sensitivity of exchange. The data are means \pm S.E. of 34/27 (control/treated) individual determinations. $^{22}\text{Na}^+$ uptake determinations also reflected the inhibitory effect. The isotope influx rates were over 60% lower in the forskolin-treated samples (Fig. 3*A*). Together, these findings imply that the C-terminal 147 amino acids are not required for the cAMP response.

Truncation at amino acid 638 had little effect on the basal functional behavior of untreated cells, but greatly reduced its responsiveness to cAMP. The pH sensitivity and cooperativity of unstimulated NHE3 Δ 638 were similar to those of the full-length NHE3 (Fig. 2, *E* and *F*; Hill coefficient ≈ 2). However, the inhibitory effect of forskolin was only marginal in this mutant, with a small divergence noted primarily at higher pH. In this figure the data are means \pm S.E. of 35/27 (control/treated) individual determinations. The reduced effectiveness of forskolin was also apparent when activity was assessed radioisotopically (Fig. 3*A*).

An additional further loss of responsiveness was noted in the NHE3 Δ 579 truncated mutant. The absolute recovery rates of these cells were low (note time scale in Fig. 2*G*), precluding detailed analysis at near neutral pH_i . Nevertheless, in the range where the measurements were reliable elevation of cAMP did not inhibit exchange. In fact, a slight stimulatory effect was recorded. The data are means \pm S.E. of 37/31 (control/treated) individual determinations. The loss of susceptibility to forskolin was also noted when $^{22}\text{Na}^+$ uptake was measured (Fig. 3*A*). The influx rates with and without forskolin were indistinguishable. In summary, the region encompassed by residues 579-684 appears to play a central role in mediating the inhibitory action of cAMP.

Structural Analysis of the Regulation of NHE3 by ATP—As described initially for NHE1, depletion of cellular ATP induces a pronounced inhibition of NHE3 activity (Fig. 4*A* and Ref. 37). In the pH_i range investigated, the rate of H^+ efflux of the full-length exchanger is almost eliminated upon metabolic depletion. The data are means \pm S.E. of 51/72 (control/treated) individual determinations. The extent of the inhibition was also apparent when measuring $^{22}\text{Na}^+$ influx (Fig. 3*B*), which showed only marginal uptake in depleted cells. As shown in Fig. 4*B*, the pH_i dependence of the exchanger undergoes a large acid shift under these conditions, with quiescence attained near pH_i 6.2. The cooperative behavior toward H^+ (Hill coefficient ≈ 2.8) was preserved following ATP depletion. It must be pointed out, however, that the need to calculate the maximal velocity by iteration in profoundly inhibited samples makes these estimates less accurate.

The inhibitory effect of ATP depletion was preserved in NHE3 Δ 684 truncated mutants. The data are means \pm S.E. of 20/20 (control/treated) individual determinations. Internally consistent results were obtained when pH_i recoveries were measured fluorimetrically (Fig. 4, *C* and *D*) and when Na^+ uptake was determined radioisotopically (Fig. 3*B*). Similarly, a profound inhibition of H^+ extrusion (Fig. 4, *E* and *H*)³ and Na^+

³ The absolute rate of the pH_i recovery for Δ 638 was found to be much higher in cells preincubated in K^+ -glutamate medium compared to HPMI (*cf.* Figs. 2*E* and 4*E*). While we do not have a definitive explanation for this observation, it is possible that this mutant is more sensitive to the presence of chloride than the other truncations.

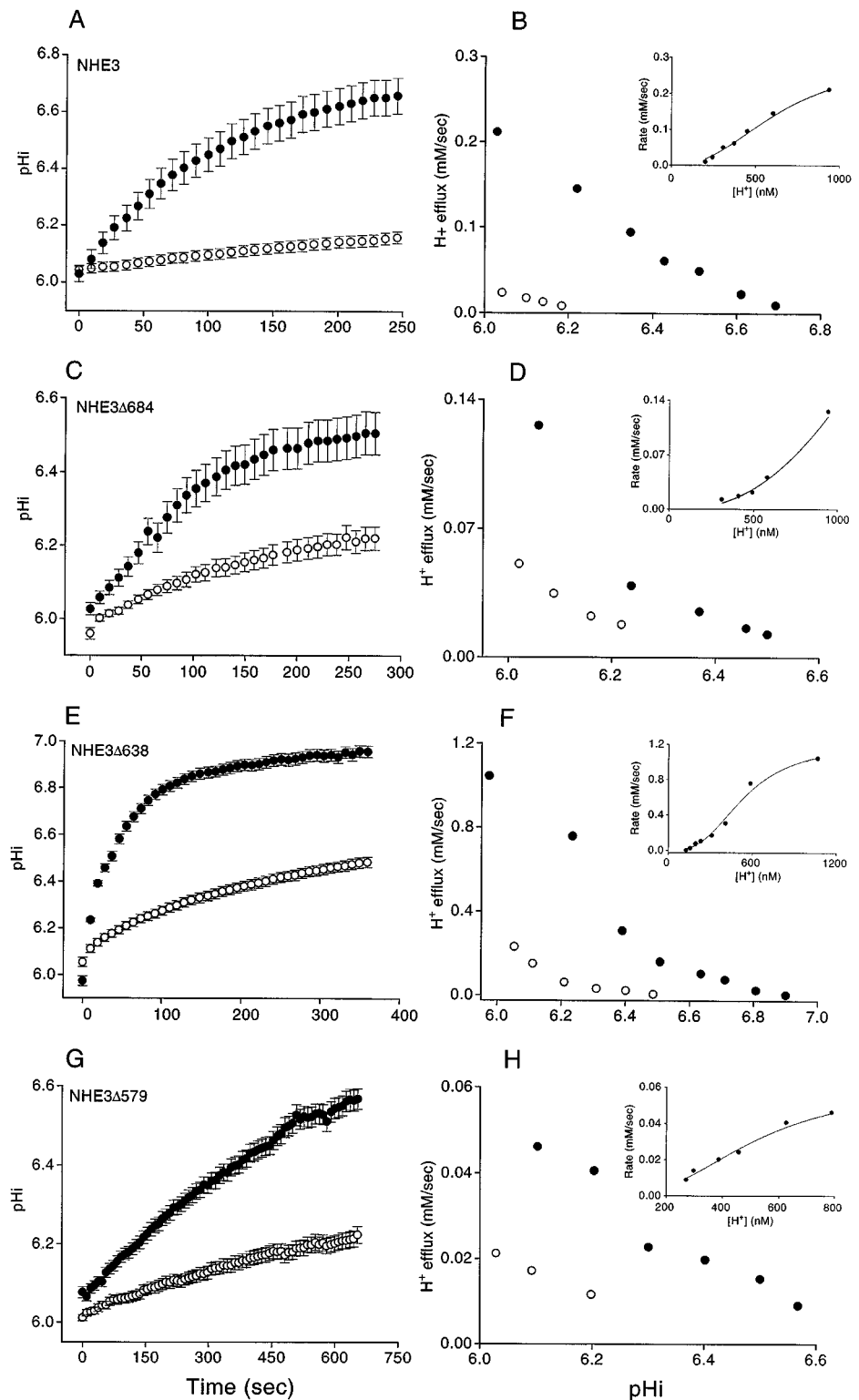


FIG. 4. Effect of ATP depletion on the activity of full-length and C-terminally truncated NHE3: pH determinations. AP-1 cells transfected with full-length NHE3 (A and B), NHE3 Δ 684 (C and D), NHE3 Δ 638 (E and F) or NHE3 Δ 579 (G and H) were loaded with BCECF in bicarbonate-free medium. The cells were acid-loaded with ammonium and treated with (open symbols) or without (solid symbols) a glucose-free medium containing 5 mM deoxyglucose plus 1 μ g/ml antimycin in order to deplete ATP during the final 10 min of incubation with the dye. Next, the pH_i recovery induced by addition of Na⁺ was measured fluorimetrically. Results in each panel are the mean \pm S.E. of at least 20 cells from three separate experiments. *Left panels* illustrate the recovery of pH_i, while the *right panels* show the pH_i dependence of the rate of H⁺ efflux, calculated from the mean Δ pH/ Δ t and the buffering power, as described under "Experimental Procedures." The *insets* show the rate as a function of [H⁺]. Data points were determined experimentally, while the line was fitted using the Hill equation (see "Experimental Procedures").

influx (Fig. 3B) was noted when NHE3 Δ 638 and NHE3 Δ 579 transfectants were subjected to the ATP depletion protocol. In these figures the data are means \pm S.E. of 61/59 and 47/29 (control/treated) individual determinations, respectively. Thus, unlike the effect of cAMP, the inhibition induced by metabolic depletion remains essentially unaffected when most of the cytosolic tail of the exchanger has been deleted.

cAMP Sensitivity and ATP Dependence of NHE3 and NHE1 Chimeric Exchangers—The behavior of full-length NHE3 and NHE1 toward challenge with cAMP is diametrically opposed;

the former is inhibited, while the latter is stimulated. This divergence provides a convenient system to test the structural conclusions derived from analysis of the truncated mutants. To this end, we constructed two chimeras; one was composed of the transmembrane domain (residues 1–504) of NHE1 and the cytosolic tail (residues 456–831) of NHE3 (designated NHE1/3; see Fig. 1). The reciprocal chimera was constructed with the transmembrane domain (residues 1–455) of NHE3 and the cytosolic tail (residues 505–820) of NHE1 (designated NHE3/1).

The expression of the chimeras was probed using antibodies directed to the cytosolic domains of either NHE1 or NHE3 (see "Experimental Procedures"). As shown earlier, anti-NHE1 antibodies recognized a wide band of approximately 110 kDa in NHE1-transfected AP-1 cells (Fig. 5A). The width of this band has been attributed to carbohydrate heterogeneity (38). The specificity of the antibody is indicated by its failure to react with NHE3 or with the NHE1/3 chimera. However, positive reactivity was detected in the NHE3/1 chimera, supporting the presence of the cytosolic domain of NHE1. Significantly, the immunoreactive band was sharper and had a molecular mass of ≈ 80 kDa, similar to that of NHE3, which unlike NHE1 is apparently not glycosylated (39). This observation is consistent with the notion that the transmembrane domain of the chimera is that of NHE3.

The immunoreactivity of the NHE3 antibody was weaker and therefore required longer exposure times. This intensified two nonspecific bands of ≈ 75 and 110 kDa, which were present in all samples but were more apparent upon extended exposure. Nevertheless, a band of 80 kDa was also discernible in NHE3 transfectants, as expected. In the NHE1/3 cells, an additional reactive band of 105–110 kDa was seen, but not in the NHE3/1 transfectant. The higher molecular mass of the

latter band suggests glycosylation of the NHE1 transmembrane domain. These findings are compatible with the predicted primary structure of the chimeras.

Additional confirmation of the composition of the chimeric constructs was obtained using amiloride and a substituted benzoyl guanidine, compound HOE 694. These inhibitors are thought to act externally on the N-terminal transmembrane region of the protein (38). More importantly, the isoforms are differentially sensitive to these drugs. As illustrated in Fig. 6, NHE1 activity was almost entirely inhibited by 1 μ M HOE 694, in agreement with the inhibitory constant ($K_i = 0.16$ μ M) reported in an earlier study (40). Similar inhibition was observed when NHE1 transfectants were exposed to 1 mM amiloride (Fig. 6). In contrast, a much higher concentration of HOE 694 (500 μ M) produced only a modest inhibition of NHE3. The nearly complete inhibition obtained with 1 mM amiloride indicates that the HOE 694-resistant component of H^+ transport is mediated by the exchanger (Fig. 6).

The inhibitor sensitivity of the NHE1/3 chimera was virtually identical to that of NHE1, implying that the two exchanger constructs share a common transmembrane domain. Conversely, the NHE3/1 chimera was resistant to 1 μ M HOE 694, resembling the behavior of NHE3. Together with the immunoblotting data of Fig. 5, these pharmacological findings confirm the composition of the chimeric constructs.

The sensitivity of the chimeras to forskolin and to ATP depletion was tested next. NHE1/3 transfectants displayed cooperativity toward H^+ (Hill coefficient ≈ 2.3) and, in the range studied, their activity was half-maximal near pH_i 6.3. Treatment of this chimera with forskolin resulted in a moderate inhibition of transport and was particularly noticeable at more acidic pH_i (Fig. 7, A and B). In this figure the data are means \pm S.E. of 24/20 (control/treated) individual determinations. The occurrence of a small yet significant inhibition was confirmed when measuring $^{22}Na^+$ uptake (Fig. 9A). In contrast, the NHE3/1 chimera was not inhibited by cAMP. Only a small, insignificant stimulation was noted at more alkaline pH_i (Fig. 7, C and D). The data are means \pm S.E. of 20/24 (control/treated) individual determinations. Again, determinations of $^{22}Na^+$ influx yielded consistent results (Fig. 9A). These findings confirm that the transmembrane region of NHE3 is not

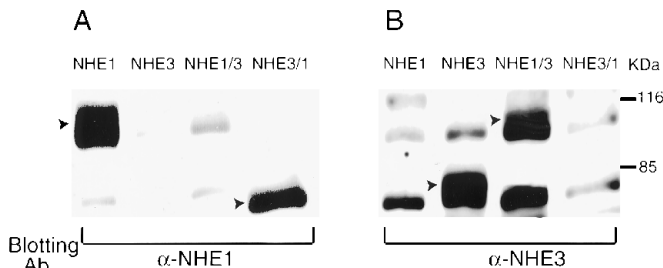


FIG. 5. Immunoblotting of the full-length NHE1 and NHE3 and chimeric NHE1/3 and NHE3/1 exchangers in AP-1 cells. Membranes (microsomal fraction) isolated from cells stably transfected with the indicated NHE were subjected to immunoblot analysis and probed with antibodies raised against NHE1 (α NHE1, panel A) or NHE3 (α NHE3, panel B). Specific immunoreactive bands are indicated by arrowheads. Lighter, nonspecific bands of ≈ 75 and 110 kDa were routinely observed in all lanes. Blots are representative of three independent experiments.

FIG. 6. Effects of amiloride and HOE 694 on the activity of full-length and chimeric exchangers. AP-1 cells transfected with full-length NHE1 or NHE3 (left column) or with the chimeric NHE1/3 or NHE3/1 (right column) were loaded with BCECF in bicarbonate-free medium. The cells were acid-loaded with ammonium and the pH_i recovery induced by addition of Na^+ was measured fluorimetrically in the presence (open symbols) or absence (solid circles) of inhibitors. The inhibitors used were amiloride (1 mM throughout; open circles) or HOE 694 (triangles). The concentration of HOE 694 used was 1 μ M for full-length NHE1 and the NHE1/3 chimera and 500 μ M for NHE3 and NHE3/1. Results are the mean \pm S.E. of at least 20 cells from three separate experiments.

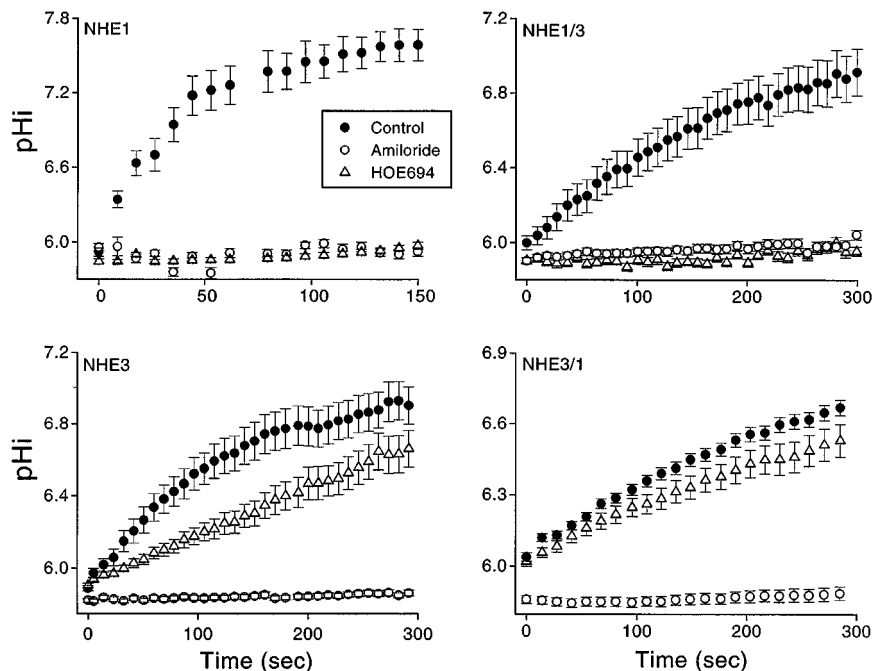


FIG. 7. Effect of forskolin on the activity of chimeric exchangers: pH determinations. AP-1 cells transfected with NHE1/3 (A and B) or NHE3/1 (C and D) were loaded with BCECF in bicarbonate-free medium. The cells were acid-loaded with ammonium and treated with (open symbols) or without (solid symbols) 10 μ M forskolin during the final 10 min of incubation with the dye. Next, the pH_i recovery induced by addition of Na^+ was measured fluorimetrically. Results in each panel are the mean \pm S.E. of at least 20 cells from three separate experiments. Other details are as described for Fig. 2.

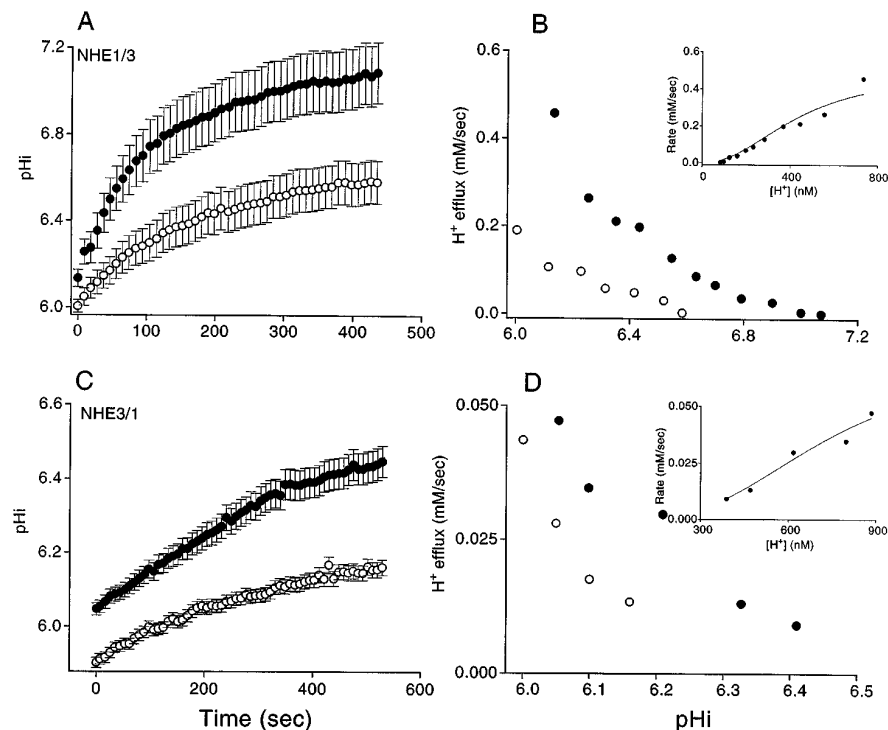
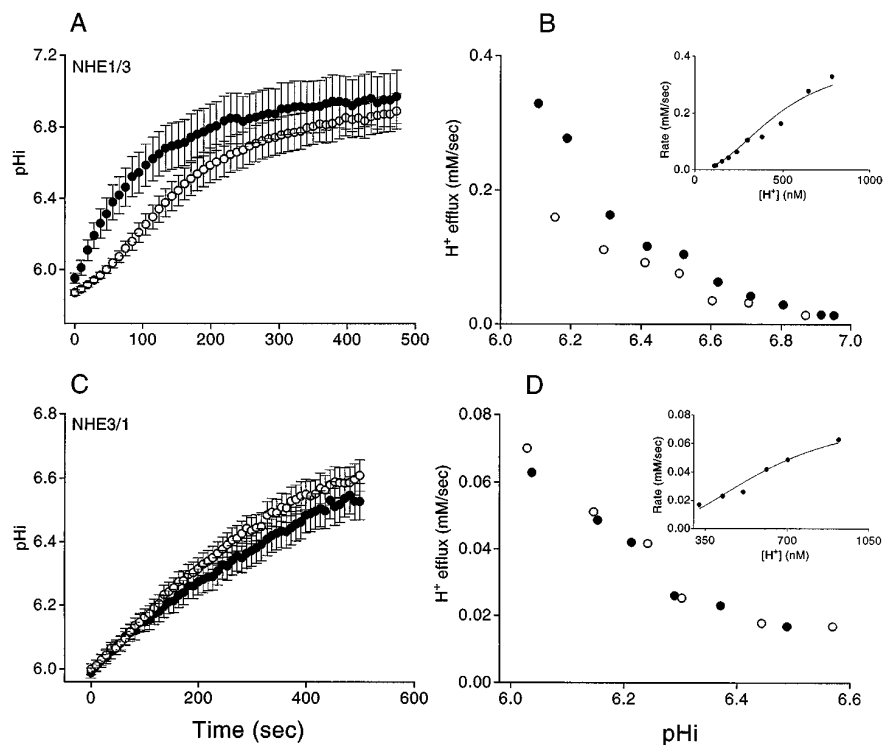


FIG. 8. Effect of ATP depletion on the activity of chimeric exchangers: pH determinations. AP-1 cells transfected with NHE1/3 (A and B) or NHE3/1 (C and D) were loaded with BCECF in bicarbonate-free medium. The cells were acid-loaded with ammonium and treated with (open symbols) or without (solid symbols) a glucose-free medium containing 5 mM deoxyglucose plus 1 μ g/ml antimycin in order to deplete ATP during the final 10 min of incubation with the dye. Next, the pH_i recovery induced by addition of Na^+ was measured fluorimetrically. Results in each panel are the mean \pm S.E. of at least 20 cells from three separate experiments. Other details are as described for Fig. 4.

sufficient to confer sensitivity to inhibition by cAMP. Moreover, the results of Fig. 7 (A and B) imply that while the cytosolic tail of NHE3 is necessary for the response, the participation of other regions of the protein is likely required to fully mimic the inhibitory effect observed in wild type NHE3.

The ATP dependence of transport by the chimeras was also tested. Both the NHE3/1 and the NHE1/3 constructs were severely inhibited when the cells were depleted metabolically. The data are means \pm S.E. of 34/42 and 22/20 (control/treated) individual determinations, respectively. The inhibition was apparent when both H⁺ extrusion (Fig. 8) and Na⁺ influx was measured (Fig. 9B). For both chimeras, inhibition appeared to

be mediated by a leftward displacement of the pH_i dependence of the rate of transport, as found for the full-length parental exchangers.

DISCUSSION

Sodium and bicarbonate reabsorption in renal and intestinal epithelial cells is mediated mainly by Na⁺/H⁺ exchange across their apical membranes. The isoform of the exchanger responsible for this exchange is believed to be NHE3. This isoform has a higher affinity for Na⁺ and is substantially less sensitive to amiloride derivatives and HOE 694 than the housekeeping NHE1 (12). The differential sensitivity to these inhibitors was

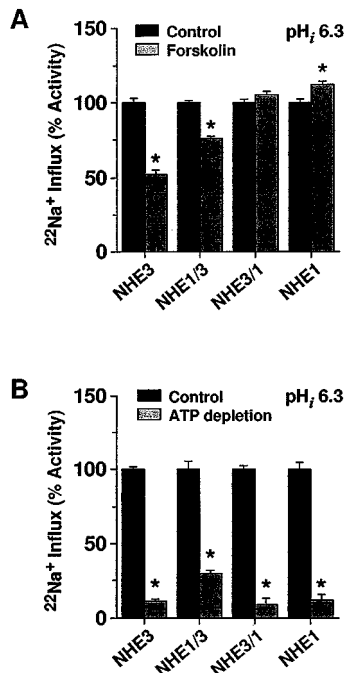


FIG. 9. Influence of forskolin and ATP-depletion on the activities of wild type and chimeric Na^+/H^+ exchangers: Na^+ uptake determinations. AP-1 cell transfectants stably expressing wild type rat Na^+/H^+ exchanger isoforms NHE1 and NHE3 and chimeric exchangers NHE1/3 and NHE3/1 were grown to confluence on 24-well plates. *A*, the effect of $10 \mu\text{M}$ forskolin on the transport activities of the exchanger was studied exactly as described in the legend to Fig. 3. Data are expressed as the mean percentage of amiloride-inhibitable $^{22}\text{Na}^+$ influx \pm S.E. ($n = 16$) from four independent experiments. *B*, the influence of cellular ATP depletion on the transport activities of the exchangers was performed as described in the legend to Fig. 3. Data are expressed as the mean percentage of amiloride-inhibitable $^{22}\text{Na}^+$ influx \pm S.E. ($n = 8$ – 12) from 2–3 independent experiments. Significance of differences between control and experimental measurements was calculated using two-tailed Student's *t* test and is indicated by an asterisk ($p < 0.001$).

confirmed in the heterologous expression model illustrated in Fig. 6. Moreover, complementary chimeras constructed with NHE3 and NHE1 indicated that the transmembrane domain suffices to dictate the degree of sensitivity to these inhibitors. This conclusion is in good agreement with earlier findings that identified unique residues within the transmembrane domain as important determinants of the affinity for amiloride and its analogs (17, 41).

Regulation by cAMP—Epithelial sodium and bicarbonate reabsorption is stringently controlled by a number of variables, including hormones that elevate the cytosolic concentration of cAMP (42). These effects are thought to be mediated by modulation of the activity of NHE3 in renal cells. Accordingly, forskolin was recently reported to inhibit amiloride-sensitive Na^+ uptake in cells transfected with NHE3 (36), an observation reproduced in the present studies (Figs. 2 and 3). It was therefore of interest to understand the structural basis of the regulation of NHE3 by cAMP. To this end, the responsiveness of a series of NHE3 chimeras and deletion mutants stably expressed in exchanger-deficient AP-1 cells to forskolin treatment was analyzed.

Inhibition of exchanger activity persisted in the NHE1/3 chimera, which contains the C-terminal cytoplasmic domain of NHE3, but was not observed in the reciprocal chimera, NHE3/1. These findings imply that the cytoplasmic domain of NHE3 is required for cAMP to exert its inhibitory action. This conclusion was confirmed by the deletion studies. Truncation of NHE3 at position 579 similarly prevented modulation by for-

skolin (Figs. 2 and 3). However, only a fraction of the cytoplasmic tail is essential for the cAMP effect, since the inhibition was intact following truncation of NHE3 at position 684. Loss of responsiveness occurred in two stages; partial effects were seen in NHE3 Δ 638, while complete unresponsiveness was apparent in NHE3 Δ 579. This behavior is suggestive of multiple target sites for cAMP, resembling the reported behavior of βNHE , the trout red cell isoform of the exchanger that is also responsive to cAMP. However, unlike NHE3, βNHE is stimulated by cAMP and has two distinct sites that are substrates for phosphorylation by PKA (43). Elimination of these sites greatly reduces but does not abolish the stimulatory effect of cAMP (44). It is noteworthy that βNHE has highest homology to the mammalian NHE1 isoform which is also stimulated by cAMP in certain cell types (26).

NHE3 differs from βNHE in that loss of responsiveness to forskolin occurs upon deletion of residues 579–684, a region that does not contain the optimal consensus sequence (R-R/K-X-S*/T*) for PKA phosphorylation (45). However, phosphorylation of this region by PKA may occur at variant sites (R-X₂-S*/T* or R-X-S*/T*) such as RLES⁶⁶¹ or RRRS⁶⁰⁵IR, which more closely resemble a classical protein kinase C consensus sequence ((R/K₁₋₃-X₂₋₀)-S*/T*-(X₂₋₀-R/K₁₋₃)) (45). In this regard, it is noteworthy that Levine *et al.* (46) recently demonstrated that the same region of NHE3 mediates inhibition of the rabbit homolog by protein kinase C, which kinetically resembles the effect of PKA. Alternatively, it is conceivable that the structure or disposition of the 579–684 domain changes upon phosphorylation of potential upstream targets (*e.g.* RRS⁵²⁰, RRGs⁵⁵², and RPS⁵⁷⁵), thereby contributing to the transduction of the inhibitory signal. It is also possible that NHE3 may not be directly phosphorylated by PKA, in which case ancillary cAMP-sensitive regulatory protein(s) may be the target of the kinase. This notion is supported by the recent discovery of a soluble cytoplasmic protein required for inhibition of Na^+/H^+ exchange by cAMP in renal brush border vesicles (47, 48). More detailed studies to test these hypotheses are currently ongoing in order to precisely delineate the molecular mechanism responsible for cAMP-dependent regulation of NHE3.

Regulation by ATP—NHE1 was initially found to be inhibited by depletion of cellular ATP, despite the passive (thermodynamically downhill) nature of the exchange process (28). Subsequent studies demonstrated that the two other isoforms analyzed thus far, NHE2 and NHE3, are similarly dependent on ATP (37). While the extent of inhibition differs somewhat between isoforms, the general pattern is comparable, suggesting similar underlying mechanisms. In accordance with this notion, the NHE1/3 and NHE3/1 chimeras were both susceptible to inhibition by metabolic ATP depletion.

Structure-function analysis of the ATP dependence was also performed by examining progressive deletions of the C-terminal tail of NHE3. In contrast to the forskolin response, the inhibitory effect of ATP depletion was preserved even in the most profound truncation studied, NHE3 Δ 579. It is concluded from these findings that the site(s) responsible for the ATP requirement are situated in the transmembrane domain or in a portion of the tail near its emergence from the membrane. It is noteworthy that ATP dependence persisted in NHE3 Δ 579 despite deletion of most consensus phosphorylation sites. For NHE1, it has been suggested that regulation by ATP is independent of changes in the phosphorylation of the exchanger itself (32). Hence, a similar mechanism may mediate the metabolic dependence of NHE3. No conventional nucleotide-binding sites are identifiable from the sequence of either NHE1 or NHE3, suggesting that perhaps associated proteins are re-

quired to confer ATP sensitivity to the exchangers. The cytoskeleton, which is drastically rearranged upon depletion of ATP, may contribute to this effect.

In summary, a similar kinetic profile is observed upon inhibition of NHE3 by cAMP and by depletion of cellular ATP. Both procedures induce a shift in the pH_i sensitivity of the exchanger to more acidic values. Nevertheless, the mutational analysis reported here implies that the mode of regulation by ATP and cAMP is unlikely to be identical. The latter required the presence of most of the cytosolic tail, while the ATP dependence persisted after more severe deletions. This points to the existence of distinct domains of NHE3 that are responsible for unique regulatory functions. More precise definition of the specific inhibitory mechanisms may require detailed information of the putative accessory proteins and their sites of interaction with NHE3.

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