A slow pH-dependent conformational transition underlies a novel mode of activation of the epithelial Na⁺/H⁺ exchanger NHE3 isoform

Hisayoshi Hayashi#, Katalin Szászi#, Natasha Coady-Osberg#, John Orlowski§, James L. Kinsella¶ and Sergio Grinstein#

#Cell Biology Programme, The Hospital for Sick Children Research Institute, Toronto, Ontario, M5G 1X8, Canada; §Department of Physiology, McGill University, Montreal, Quebec, H3G 1Y6, Canada and ¶Laboratory of Cardiovascular Science, Gerontology Research Center, NIA, NIH, Baltimore, MD 21224, USA

This study was supported by the Canadian Institutes of Health Research (CIHR) and the Kidney Foundation of Canada

Support by a CIHR fellowship

J.O. is an Investigator of the CIHR

S.G. is an International Scholar of the Howard Hughes Medical Institute and is the current holder of the Pitblado Chair in Cell Biology at The Hospital for Sick Children. Cross-appointed to the Department of Biochemistry, University of Toronto.

Running Title: slow activation of Na⁺/H⁺ exchange

Keywords: NHE; antiport; pH regulation; cytoskeleton, F-actin; LLC-PK₁ cells

Correspondence to: Sergio Grinstein

Cell Biology Program, Hospital for Sick Children Research Institute
555 University Avenue
Toronto, Ontario
M5G 1X8, CANADA
Phone: 416 813 5727
FAX: 416 813 5028
Email: sga@sickkids.ca
SUMMARY

Allosteric control of Na⁺/H⁺ exchange by intracellular protons ensures rapid and accurate regulation of the intracellular pH. While this allosteric effect was heretofore thought to occur almost instantaneously, we report here the occurrence of a slower secondary activation of NHE3, the epithelial isoform of the exchanger. This slow activation mode developed over the course of minutes and was unique to NHE3 and the closely related isoform NHE5, but was not observed in NHE1 or NHE2. Activation of NHE3 was not due to increased density of exchangers at the cell surface, nor was it accompanied by detectable changes in phosphorylation. The association of NHE3 with the cytoskeleton, assessed by its retention in the detergent-insoluble fraction, was similarly unaffected by acidification. In contrast to the slow progressive activation elicited by acidification, deactivation occurred very rapidly upon restoration of the physiological pH. We propose that NHE3 undergoes a pH-dependent slow transition from a less active to a more active state, likely by changing its conformation or state of association.
INTRODUCTION

Na\textsuperscript{+}/H\textsuperscript{+} exchangers (NHE) catalyze the electroneutral counter-transport of Na\textsuperscript{+} for H\textsuperscript{+} across biological membranes (for review, see refs. 1-3). To date, seven different NHE isoforms have been cloned, all of which are integral membrane proteins with multiple transmembrane domains and an extensive cytosolic carboxy-terminal domain. NHE1 and NHE3 are by far the most widely studied isoforms. The former is expressed ubiquitously and, by mediating Na\textsuperscript{+}/H\textsuperscript{+} exchange across the plasma membrane, contributes to the regulation of cytosolic pH and cell volume (2). NHE3 is found in the brush border of epithelial cells in the kidney and gastrointestinal tract and is involved in the transepithelial (re)absorption of NaCl, and, indirectly, HCO\textsubscript{3}\textsuperscript{-} and water (4).

Because Na\textsuperscript{+}/H\textsuperscript{+} exchange is electroneutral with a 1:1 stoichiometry, thermodynamic equilibrium is predicted to be attained when \([\text{H}^+]_i / [\text{H}^+]_o = ([\text{Na}^+]_i / [\text{Na}^+]_o) \cdot \text{Ke}\), where Ke is the equilibrium constant and the subscripts \(i\) and \(o\) refer to intra- and extracellular, respectively (5). In almost all cells, a steep inwardly directed Na\textsuperscript{+} gradient is maintained across the plasma membrane due to extrusion of Na\textsuperscript{+} by the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase. Based on the concentrations recorded in most cases ([Na\textsuperscript{+}]\textsubscript{o} being at least ten times higher than [Na\textsuperscript{+}]\textsubscript{i}) it is apparent that NHE could drive the intracellular pH (pHi) at least 1 unit above the external pH. Nevertheless, pHi rarely exceeds and is usually lower than the extracellular pH, because NHE becomes virtually quiescent at pHi \(\approx\) 7.2. This behavior has been attributed to the existence of an allosteric, pH-sensitive site on the cytosolic aspect of the NHE. The set point at which quiescence is dictated by this allosteric or "modifier" site is presumably intended to stabilize pHi.
near the physiological optimum level and to preclude deleterious alkalinization (6,7).

While little is known about the molecular identity of the modifier site, the pH dependence of activation of NHE suggests that the rate of transport is modulated by protonation of one or more side chains of the protein. It has been postulated that the resulting change in surface potential may alter the local concentration of H⁺ (equivalents) and thus their availability for the exchange reaction. Because protonation/deprotonation of side chains is expected to occur almost immediately after pH is altered, the effect of the modifier site has been tacitly assumed to occur instantaneously, providing rapid feedback and thereby accurate regulation of pHi.

In the course of experiments using isolated brush border membranes at subphysiological temperatures, we detected significant hysteresis in the inactivation of NHE3 (preliminary results are described in (8)). This slow responsiveness, which developed over the course of minutes, cannot be easily reconciled with the simple side-chain protonation model described above. We therefore decided to investigate whether slow activation and/or inactivation transitions are observed in intact cell systems and to examine the underlying mechanism. To this end we used the porcine kidney LLC-PK₁ cells, which are known to express endogenous NHE3 (9). In addition, we compared the behavior of several isoforms of NHE expressed heterologously in antiport-deficient Chinese hamster cells. Our results revealed a novel mode of regulation that is unique to NHE3 and the related isoform, NHE5, and likely involves large conformational changes and/or interaction with other molecules.
MATERIAL AND METHODS

Materials and Solutions. Nigericin, the acetoxymethyl ester of 2’, 7’- bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and Alexa Fluor 488-conjugated donkey anti-mouse antibody were obtained from Molecular Probes, Inc. (Eugene, OR). Mouse anti-HA antibodies were from BabCo (Berkeley, CA). 125I-labeled goat IgG and 32P-orthophosphate were from ICN (Costa Mesa, CA). Isotonic Na+ medium contained (in mM): 140 NaCl, 3 KCl, 1 MgCl2, 1 CaCl2, 20 Hepes; pH was adjusted to 7.4 with Tris at room temperature. The Na+-free solution was prepared by equimolar substitution with N-methyl-D-glucamine. Isotonic K+-rich medium had the same composition as Na+-rich medium, except that NaCl was replaced by KCl.

Cells. LLC-PK1 cells were obtained from the American Type Culture Collection. For surface detection and immunoprecipitation experiments LLC-PK1 cells were transfected with wild-type NHE3 containing three tandem copies of the influenza virus hemagglutinin (HA) epitope, YPYDVPDYAS, in the first extracellular loop - between amino acids Arg38 and Phe39 - as described previously (13). LLC-PK1 cells were transfected using FuGene6, as described by the manufacturers. For selection of stable lines, cells were cotransfected with the pCMV plasmid, which contains the aminoglycoside phosphotransferase gene that confers resistance to G418, and were selected by limiting dilution cloning in the presence of 500 µg/mL G418 and screened by immunofluorescence for expression of the HA-tagged NHE3 (NHE3’38HA3). Cells were maintained in 1:1 DMEM/F-12 with 10% fetal bovine serum in an atmosphere containing 5% CO2.
AP-1 is a cell line devoid of endogenous Na\(^+\)/H\(^+\) exchange activity that was isolated from WT5 Chinese hamster ovary cells as previously described (10). NHE cDNA constructs were transfected into AP-1 cells by the calcium-phosphate-cDNA co-precipitation technique and stable clones were selected for survival by imposing acute NH\(_4\)Cl-induced acid loads (11,12). Cells were maintained in \(\alpha\)-minimal essential medium with 10% fetal bovine serum in an atmosphere containing 5% CO\(_2\).

**Measurement of Na\(^+\)/H\(^+\) activity.** Cytosolic pH (pHi) was measured by microphotometry of the fluorescence emission of BCECF using dual wavelength excitation. The activity of the Na\(^+\)/H\(^+\) exchanger was determined as the rate of Na\(^+\)-induced pH recovery after acid loading with NH\(_4\)Cl. LLC-PK\(_1\) cells grown to confluence on 25-mm glass coverslips were incubated with 2 \(\mu\)g/mL of BCECF acetoxymethyl ester plus 15 mM NH\(_4\)Cl and after 10 min the cells were washed with Na\(^+\)-free solution. Na\(^+\)/H\(^+\) exchange was then initiated by reintroduction of extracellular Na\(^+\) at the indicated time. Clusters of the cells adherent to the coverslip were selected for pH measurements, while cells that formed domes were avoided, because in these the Na\(^+\)-induced pH recovery likely occurred via basolateral NHE. In order to minimize the consequences of dye leakage from the cells, the chamber was continuously perfused at 1.0 mL/min using a gravity-driven system. The fluorescence of BCECF was measured and calibrated as described previously (13). The cellular buffering capacity was determined from the pH changes observed in response to NH\(_4\)Cl pulses imposed at various pH levels. H\(^+\) (equivalent) flux rates were calculated by multiplying the rate of pH recovery times the
buffering capacity, at the corresponding pH values. All procedures were performed at room temperature.

**Intracellular Na+ content manipulation and determination.** LLC-PK₁ cells were plated onto 6-well plates and grown to subconfluence. We did not use confluent cells because of potential trapping of extracellular fluid under domes. Intracellular Na⁺ depletion was accomplished by incubating the cells in Na⁺-free solution. Intracellular Na⁺ content was determined by flame photometry (Photometer model 443, Instrumentation Laboratory, Lexington, MA) using Li⁺ as an internal standard. After intracellular Na⁺ depletion, the cells were washed with an ice-cold medium containing 300 mM sucrose and 10 mM Hepes-Tris, pH 7.4. The cells were scraped off with a rubber policeman into Li⁺ standard solution (Instrumentation Laboratory), the samples were frozen and thawed, insoluble material was sedimented and the supernatant was used for measurement. Intracellular Na⁺ content was normalized by the number of cells used. Cells were counted electronically with a Coulter Counter (Coulter Electronics, Hialeah, FL).

**Immunofluorescence.** LLC-PK₁ cells stably expressing NHE3’38HA₃ were plated onto glass coverslips and grown to confluence. The cells were rinsed with PBS and, where indicated, acidified for 5 min with NH₄Cl as described above, before fixing for 20 min using 8% paraformaldehyde in ice-cold PBS. Following fixation, the cells were washed 3 times with PBS and incubated with 100 mM glycine in PBS for 10 min. The cells were next pre-blocked with 5% skimmed milk in PBS for 30 min, then incubated with monoclonal anti-HA antibody
(1:1000 dilution) for 1 h. After washing 3 times to remove unbound antibody, the cells were incubated with Alexa 488-conjugated donkey anti-mouse antibody (1:1000 dilution) for 1 h and the coverslips mounted onto glass slides with DAKO mounting medium (DAKO Corp., Carpinteria, CA).

Detergent extraction and Immunoblotting. The detergent solubility of NHE3’38HA3 was assessed in transfected LLC-PK₁ cells cultured in 35 mm dishes and grown to confluence. Where indicated, the cells were first acid-loaded to pH 6.4 as described above. The cells were then rinsed twice with ice-cold medium and extracted with 1 mL of ice-cold lysis medium containing 150 mM NaCl, 25 mM HEPES, 25 mM MES, 0.5 mM EGTA, 8% protease inhibitor cocktail (Complete; Roche) plus 2 µM pepstatin and either 0.1% Triton X-100 or 1% digitonin. The pH of the media was adjusted to either 6.4 or 7.4 as indicated. After swirling on ice for 6 min on an orbital shaker, the supernatant was removed and saved. The adherent material was washed with ice-cold PBS and scraped off with a rubber policeman into 1 mL of ice-cold PBS containing 0.1% SDS and 8% protease inhibitor cocktail plus 2 µM pepstatin. Protein concentration was measured by the method of Ghosh S. et al (14), quantifying by densitometry (AlphaImager, AlphaInnotech, U.K.). Following addition of 5x concentrated Laemmli sample buffer, samples were subjected to SDS-PAGE and transferred onto nitrocellulose filters. Blots were blocked with 5% milk and exposed to the primary antibody (1:5000 anti-HA or 1:250 anti-actin antibodies). Horseradish peroxidase-conjugated secondary antibody was applied (1:5000), and immunoreactive bands were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech) and were quantified by densitometry.
Quantification of surface NHE3. To quantify surface NHE3 expression, LLC-PK1 cells stably expressing NHE3'-3HA3 were plated onto 6-well plates and grown to confluence. The cells were rinsed with PBS and, where specified, acidified for 5 min with NH4Cl as above. The cells were next washed with ice-cold PBS, pre-blocked with 10% goat serum in PBS for 30 min on ice, and then incubated for 45 min on ice with anti-HA antibody (1:500 dilution) in medium with 10% goat serum. After washing 4 times to remove unbound antibody, the cells were incubated with 125I-labeled goat anti-mouse IgG (0.4 µCi/well) in medium with 10% goat serum on ice. After 60 min, cells were washed 4 times with ice-cold PBS to remove unbound radioactivity. The radiolabeled antibodies were eluted with 1 mL of 2 M formic acid and radioactivity was counted using a gamma counter (1282 Compu Gamma, LKB, Finland). Non-specific binding of the radiolabeled antibodies, assessed by omitting primary antibodies, was subtracted.

Assessment of NHE3 Phosphorylation. LLC-PK1 cells stably expressing NHE3'-3HA3 grown to confluence on 15-cm dishes were labeled for 4 h by incubation with 4 mL of phosphate-free DMEM medium containing 0.4 mCi of [32P]-orthophosphate. After labeling, the cells were washed three times with PBS and, where specified, acidified with NH4Cl for 5 min as above. To enrich the preparation in NHE3 prior to immunoprecipitation we prepared brush border membrane vesicles from the labeled LLC-PK1 cells by the method of Brown et al. (15), with minor modifications. The cells were scraped off with a rubber policeman into 1 mL of ice-cold medium containing 300 mM mannitol, 5 mM NaF, 2 mM Na3VO4, 1% protease inhibitor (Sigma) and 10 mM HEPES-Tris, pH 7.4 and homogenized with a bath sonicator (Fisher model
300, setting 60) for 5 min. Next, MgCl2 was added to a final concentration of 10 mM followed by incubation for 30 min on ice and centrifugation for 15 min at 2,300 x g. The supernatant was collected and subjected to centrifugation for 22,200 x g. The resulting pellet containing the brush border vesicles was resuspended in 1 mL of RIPA buffer (150 mM NaCl, 0.1% SDS, and 1% Triton X-100, 50 mM NaF, 2 mM Na3VO4, 1% protease inhibitor (Sigma) and 20 mM Tris-HEPES at either pH 7.4 or 6.4) by drawing through a 26 gauge needle, and shaken on a rotating rocker for 30 min at 4°C. After centrifugation at 88,800 x g for 30 min to remove insoluble cellular debris, the supernatant was added protein G-Sepharose beads (Pierce) which were pre-conjugated with anti-HA monoclonal antibody, and the suspension was shaken for 2 h at 4°C. The beads were spun down and washed eight times with RIPA buffer. Immunoprecipitated proteins were eluted by incubation in 50 µL of Laemmli sample buffer at 65°C for 15 min. An aliquot of 40 µL of each sample was analyzed by SDS-PAGE and electrophoretically transferred to a Trans-Blot membrane (Bio-Rad, CA). The relative amounts of NHE3 in the samples were determined by ECL and quantified using the AlphaImager. Once the enhanced chemiluminescence decayed, 32P signals from the same membranes were subsequently measured using a PhosphorImager and quantified using ImageQuant (Molecular Dynamics).

**Statistical analysis.** Experimental values are given as the mean ± S.E.M of the indicated number of determinations. Comparisons between two groups were made by either unpaired or paired Student’s t tests, as appropriate.
RESULTS

Time dependence of NHE3 activation. The time dependence of the activation of NHE3 was studied in LLC-PK₁ cells by monitoring the Na⁺-induced recovery of pHᵢ following an acid load. Controlled cytosolic acidification was imposed by the NH₄Cl (15 mM) pre-pulse technique (16) and Na⁺/H⁺ exchange was initiated when desired by addition of 140 mM Na⁺ to the medium. It is noteworthy that: i) in the absence of Na⁺, NHE-independent pHᵢ recovery was negligible (0.003 pH/min) and ii) the acid loading was essentially complete at the time of addition of Na⁺ (e.g. Fig. 1A) so that the rate of pH recovery was uncontaminated by an opposing tendency to acidify due to residual intracellular ammonium. Only in the case of the measurements made 1 min after removal of ammonium was a small acidification still apparent. This rate of acidification was measured in parallel experiments (0.011 mM/sec) and was added to the determinations of NHE based on the net rate of alkalinization.

Two measures were taken to ensure that the activity of NHE3 was assessed without contribution from NHE1, which is expressed in the basolateral membrane of LLC-PK₁ cells (17). First, the cells were grown to confluence and Na⁺ was added only apically. Secondly, 100 µM amiloride was added to selectively inhibit NHE1 (18).

Under the conditions used (pHᵢ 6.53 ± 0.03, n =25), the Na⁺-induced pHᵢ recovery was slow when the cells were acidified for only 1 min. However, the rate of alkalinization increased gradually as the period of acidification was lengthened (Fig. 1A). The open symbols in Fig. 1C summarize the results of 6 experiments: the rate of Na⁺/H⁺ exchange increased progressively up to 5 min and decreased slowly thereafter. The maximal rate observed was two-fold greater than
the rate measured at 1 min. Shorter periods were not analyzed because the acid loading procedure was still ongoing before 1 min (see above).

A limited number of experiments were also performed using opossum kidney (OK) cells, with similar results (not shown). These findings indicate that the slow activation of Na⁺/H⁺ exchange is a widespread phenomenon and suggest that the observations in LLC-PK₁ cells were attributable to NHE3, since OK cells express NHE3 but not NHE1 (19,20).

The faster rate observed after prolonged acidification is likely to reflect increased activity of NHE3. However, it is conceivable that the cytosolic buffering power diminishes over time after acidification, resulting in a faster recovery at constant NHE activity. The latter possibility was explored using an exogenous ion exchanger. As shown in Fig. 1B, the pHᵢ recovery induced by nigericin and high K⁺ solution was similar whether the ionophore was added 1 or 5 min after acidification was imposed. The nigericin induced recovery averaged 0.05 ± 0.01 and 0.04 ± 0.01 at 1 and 5 min, respectively (solid symbols in Fig. 1C). These observations rule out a reduction in buffering power and also other possible time dependent artifacts in the recording system.

**Intracellular Na⁺ depletion does not contribute to increasing of NHE3 activity.** Imposition of an acid load of variable duration required incubation in Na⁺-free medium for different periods of time. This may have resulted in variable intracellular Na⁺ contents at the time of assaying NHE. The observed time-dependent increase in activity may therefore have resulted from an increased driving force for forward Na⁺/H⁺ exchange, or from stimulation of a Na⁺-sensitive G protein, as has been invoked for the regulation of NHE1 (21). To analyze this possibility, cells were
initially pre-incubated in Na+-free medium for 10 min and then subjected to acid load and
challenged with extracellular Na+. As illustrated in Fig. 2A, the rate of recovery after a 1 min
acidification was not significantly different from that recorded in control cells in Fig. 1 (0.035 ±
0.003 mM/sec vs. 0.034 ±0.003 mM/sec, respectively, p=0.87). More importantly, the time-
dependent acceleration of NHE3 activity persisted in the Na+-depleted cells: the rate of pH
recovery was nearly 3-fold higher after 5 min than after 1 min (Fig. 2A-B).

To confirm that depletion of Na+ was not the mechanism underlying stimulation, we
measured the intracellular content of this cation by flame photometry under all the conditions
used. Omission of extracellular Na+ prior to the acid load reduced intracellular Na+ by 45%
(from 44 to 20 mM). The magnitude of the depletion was not significantly different (52 ± 5 %
vs.45 ± 8 %; p=0.61) when Na+ removal was extended for 15 min, indicating that no substantive
further depletion occurred during the acid loading period in Fig. 2. These results indicate that
intracellular Na+ depletion does not contribute to the time-dependent activation of NHE3.

\textit{pH}_i \textit{dependence of the activation of NHE3.} To analyze the effect of pH\textsubscript{i} on the kinetics of
NHE3 activation (Fig. 3), various concentrations of NH\textsubscript{4}Cl were used during the pre-pulse. The
time dependence of the activation was recorded at all the pH\textsubscript{i} levels that we measured (between
6.5 - 7.5) and in all cases the differential between 1 and 5 min was e2 fold. Interestingly, when
plotting efflux rate \textit{vs.} [H\textsuperscript{+}]\textsubscript{i} (Fig. 3C), the data conformed to Michaelis-Menten kinetics when
the acidification was imposed for 5 min (R\textsuperscript{2}= 0.781), but the fit was poorer after 1 min (R\textsuperscript{2} =
This observation suggests that a more complex process, involving the time-dependent activation of NHE3 is ongoing at 1 min. If activation is complete by 5 min, only the expected pseudo-first order kinetics is noted.

*The number of surface NHE3 does not change during acidification.* In native, as well as in heterologous expression systems, NHE3 is present in at least two subcellular compartments: the plasma membrane and an endomembrane vesicular compartment which includes subapical/recycling endosomes (13,22,23). In opossum kidney cells, it was shown that chronic exposure to acidic solutions increases the activity of NHE3 and that such increase is accompanied by mobilization of endomembrane NHE3 to the cell surface (24). It was therefore conceivable that the number of surface NHE3 may change also during acute intracellular acidification. To assess this possibility, the abundance of cell surface NHE3 was quantified by two methods. First, we generated a stable clone of LLC-PK₁ cells expressing an epitope-tagged form of NHE3. The epitope was placed in an extracellular loop, to enable detection of surface exposed NHE3 in intact cells (see ref. (25) for details). Immunostaining and fluorescence imaging analysis of intact (non-permeabilized) cells before and after acidification revealed no visible differences in the density or distribution of NHE3 (Fig. 4A). To more accurately quantify surface NHE3 we used ¹²⁵I-labeled antibodies. As shown in Fig.4B, the surface density of NHE3 did not change following acidification. These results imply that the intrinsic activity, and not the number of exchangers, is increased when acid loading is sustained for 2-5 min.

*Phosphorylation of NHE3 does not change during acidification.* It is well established that
NHE3 can become phosphorylated on Ser residues and that phosphorylation is accompanied by changes in transport activity (26-29). In addition, Tyr phosphorylation may play a role in NHE3 regulation, since stimulation of exchange by chronic acidification is associated with activation of c-Src (30). To define whether the more acute acid-induced stimulation reported here was similarly attributable to phosphorylation, cells were preincubated with $^{32}$P-orthophosphate and subjected to acidification. The cells were then immediately lysed in the cold and NHE3 was immunoprecipitated. Quantitation of the radioactivity following analysis by SDS-PAGE revealed that the extent of phosphorylation of NHE3 was essentially identical before and after acidification for 5 min (Fig. 4B). Therefore, a mechanism other than direct phosphorylation must account for the observed time-dependent activation of NHE3.

**Biochemical assessment of the interaction between NHE3 and the actin cytoskeleton.** We previously demonstrated a functional interaction between NHE3 and the actin cytoskeleton in AP-1 cells (31). In those studies the activity of the antiporter was modulated by the extent of actin polymerization, raising the possibility that the cytoskeleton may contribute to the pH-dependent effects reported here. To test this possibility, we examined the association of NHE3 with the detergent-insoluble fraction of LLC-PK1 cells, which contains (part of) the actin cytoskeleton. Cells were either left untreated or were acid-loaded to pH 6.4 as described for the determinations of exchange activity. Next the cells were extracted in detergent at pH 6.4 or 7.4 and the soluble and insoluble fractions of NHE3 were quantified by immunoblotting (Fig. 5A). For reference, the total amount of protein and of actin solubilized were also quantified in the extracts. When using Triton X-100, only a fraction of the NHE3 was solubilized, regardless of
the pH of the extraction solution (27 ± 2% vs. 31 ± 1%, pH 7.4 and pH 6.4, respectively). This implies that the majority of the exchangers are constitutively associated with the cytoskeleton. Under the conditions used, the Triton-insoluble fraction may also include glycolipid-enriched microdomains, known as rafts (32). It was therefore conceivable that the lipid partitioning of NHE3, as opposed to its cytoskeletal interactions, dictated the insolubility of the exchanger. This alternative explanation was tested using digitonin, another non-denaturing detergent which, unlike Triton, preferentially interacts with and extracts cholesterol from biological membranes. Digitonin solubilized a slightly larger fraction of NHE3 (42 ± 8%) than did Triton X-100, favoring the notion that the exchanger is rendered insoluble primarily through its interaction with the cytoskeleton.

Importantly, the fraction of the exchangers that were extractable by Triton was not significantly altered when the cells were acidified before and/or during the extraction procedure (Fig. 5A and B). The amounts of protein and actin solubilized in both instances were also comparable (Fig. 5B), and similar results were obtained with digitonin (not shown).

Jointly, these results suggest that the interaction of NHE3 with the cytoskeleton is not grossly altered by the acid-loading procedure. It therefore appears unlikely that the cytoskeleton plays a role in the time- and pH-dependent activation of the exchanger. This concept was further supported by observations made in cells transfected with NHE3638, a truncated version of NHE3 that was shown earlier to be insensitive to cytoskeletal alterations (31). As shown in Fig. 5C, the rate of Na+/H+ exchange in these transfectants was accelerated when the acidification period was extended, as shown above for the endogenous (full-length) exchanger of epithelial cells. The rate of recovery was nearly 3-fold greater after 3 min than after 1 min (Fig. 5D). These
observations strongly suggest that the cytoskeleton is not the main determinant of the time-dependent activation of NHE3.

Kinetics of NHE3 inactivation following slow activation. We next assessed the rate at which NHE3 inactivates following slow activation by a sustained acid load. The cells were first acidified for 5 min to activate NHE3 fully, omitting Na⁺ from the medium to preclude pH recovery during this period. Next, the cytosolic pH was increased as fast as possible by reintroducing an amount of NH₄Cl, found empirically to elevate pH to near baseline (pre-acid loading) levels. Finally, Na⁺ was added to the bathing medium to initiate Na⁺/H⁺ exchange. Re-introduction of Na⁺ failed to elevate pH beyond the resting levels (Fig. 6), implying that NHE was not more active than it was prior to acid loading. This indicates that NHE3 inactivated rapidly (within 1 min) after restoration of neutral pH, so that inactivation appears to be much faster than the slow activation process, which develops over 5 min.

Is slow activation an intrinsic property of NHE3? It could be argued that the slow activation of NHE3 reported above is a property of the LLC-PK₁ cells in which its activity was analyzed, rather than being characteristic of this isoform. This concern was addressed by studying the behavior of NHE3 expressed heterologously in AP-1 cells. AP-1 cells, a sub-line of Chinese hamster ovary cells, are devoid of endogenous NHE activity (10) and are therefore well suited to analyze the activity of heterologously expressed exchangers. As shown in Fig. 7B, a clear time-dependent activation was noted also in NHE3-transfected AP-1 cells. This finding implies that the slow pH-dependent activation of NHE3 is an intrinsic property of this isoform that is
manifested in both epithelial and non-epithelial contexts.

Time course of activation of various NHE isoforms. It was of interest to establish whether, like NHE3, other NHE isoforms similarly undergo a slow activation following acidification. To this end, we analyzed the behavior of AP-1 cells transfected with defined isoforms of the exchanger. As above, AP-1 cells enabled us to unambiguously attribute the exchange activity to the heterologously transfected NHE isoform of interest. Typical results are shown in Fig. 7A, illustrating the recovery mediated by NHE1 following acid-loading for either 1 or 3 min. As summarized in Fig 7B, the rate of Na⁺/H⁺ exchange was not affected by the duration of the acid load. Similar results were obtained in AP-1 cells transfected with NHE2. NHE5, which amongst the known NHE isoforms shares the greatest homology with NHE3 (33), displayed a significantly greater activity after 3 min than after only 1 min of acidification, although the difference was less striking than in the case of NHE3. Thus, the time and pH-dependent activation of NHE is isoform specific, occurring only in NHE3 and the closely related NHE5, but not in the case of NHE1 and NHE2.
DISCUSSION

Our results revealed the existence of a slowly developing activation of NHE3, which required 4-5 min to attain completion. This comparatively slow transition is unlikely to reflect the rate of protonation of dissociable groups of the side chains of NHE3, and may instead result from sizeable conformational changes of the exchanger and/or of ancillary proteins that regulate its activity. Such structural changes could either induce or stabilize an active conformation of the antiporter. As is widely accepted in the case of ion channels, NHE is proposed to exist in an inactive (or poorly active) form (I) and one or more active configurations (A). Protonation would facilitate this transition by either increasing the rate of conversion (k₁) or by inhibiting its rate of reversal (k₋₁). To the extent that other isoforms show a pH dependent activation, but not the slow transition displayed by NHE3 and NHE5, we suggest that two activated states (A₁ and A₂) exist for the NHE3 and 5 (equation 1), but only one for the other isoforms:

\[
\begin{align*}
I & \overset{k_1}{\longrightarrow} \overset{k_2}{\longleftarrow} A_1 \\
& \overset{k_-1}{\longleftarrow} \overset{k_-2}{\longrightarrow} A_2
\end{align*}
\]

The uniquely slow activation from A₁ to A₂ would be dictated by k₋₂, which is presumably lower than k₁ and therefore limiting to the overall activation process. Note that only k₁ (and/or k₋₁) need to be pH sensitive, which would agree with our observation that the course of the slow transition was not markedly different when the extent of the acid loading was varied.

The molecular nature of the event mediating the slow activation of NHE3 remains to be completely defined. Kinsella et al. (8) explored earlier the possibility that large multimeric
complexes of exchangers may form upon acidification. However, cross-linking experiments failed to show evidence that higher order aggregates are formed. Alternatively, when the cytosol is acidified, NHE3 may partition preferentially into detergent-insoluble lipid rafts, as has been suggested for apically targeted proteins in the trans-Golgi network (32). This explanation was rendered unlikely by the observation that the solubility of NHE3 in digitonin, a cholesterol-extracting detergent that effectively disrupts rafts, was similar in control and acid-loaded cells.

A functional relationship between NHE3 and the actin cytoskeleton had been suggested before (31), based on the inhibitory effects of cytoskeletal disruption. Our data showing that most of the NHE3 in LLC-PK1 cells was not solubilized by either Triton X-100 or digitonin (Fig. 5) is consistent with association of the exchangers with the actin cytoskeleton. Accordingly, pretreatment of the cells with Clostridium difficile toxin B, which inhibits Rho-family GTPases and causes microvillar retraction (34,35), markedly increased the fraction of NHE3 that is solubilized by Triton (H. Hayashi, unpublished observations). The interaction of the antiporter with the cytoskeleton, however, was not noticeably altered by cytosolic acidification (Fig. 5A-B). Moreover, a truncated form of NHE3 that lacks modulation by the cytoskeleton nevertheless displayed the time- and pH-dependent stimulation (Fig. 5C-D). We therefore believe that the activation of the exchanger is not caused by drastic alterations in its cytoskeletal anchorage.

We tentatively favor the notion that the A1 to A2 transition reflects a change in the conformation of the exchanger. The comparatively slow kinetics of the transition implies that the event requires a large activation energy, suggesting that the conformational change is drastic. Future structural studies should be able to test this prediction.

The observation that NHE3 undergoes a slow activation transition adds to the complexity of
regulation of this isoform by H+. In addition to the rapid activation, noticeable in all isoforms within seconds of acid loading (likely reflecting the I to A₁ transition) and the slower phase described herein (postulated to be the A₁ to A₂ transition), a much slower stimulation of NHE3 by chronic acidification was described earlier (36-38). This acceleration of transport differs from the one described herein in that it requires several hours to develop, involves de novo synthesis of mRNA and proteins (38) and is accompanied by increased exocytosis of intracellular NHE3 molecules.

Why are so many forms of regulation of NHE3 by acid required? Rapid responses, occurring within seconds are necessary for the acute and fine regulation of the intracellular pH. Those developing over minutes may be intended to compensate for acute variations in systemic pH and bicarbonate concentration. More severe and chronic challenges likely require further and more sustained increases in the rate of transport. This is accomplished by biosynthetic means, at the expense of reduced response time. In this regard, it is noteworthy that the secondary activation described in this report appears to revert rapidly upon restoration of the normal cytosolic pH (Fig. 6), conferring to the system effective feedback properties. In contrast, the insertion into the brush border of the extra transporters synthesized and mobilized during chronic acidosis would show greater hysteresis, leading to a potentially dangerous overshoot in the rate of acid extrusion when the normal pH is restored. Thus, the various levels of regulation are not redundant, but complementary and individually well suited for fast or sustained challenges to the cellular and systemic pH and bicarbonate homeostasis.
REFERENCES

1. Orlowski, J., and Grinstein, S. (1997) J Biol Chem 272, 22373-22376

2. Wakabayashi, S., Shigekawa, M., and Pouyssegur, J. (1997) Physiol Rev 77, 51-74

3. Counillon, L., and Pouyssegur, J. (2000) J Biol Chem 275, 1-4.

4. Schultheis, P. J., Clarke, L. L., Meneton, P., Miller, M. L., Soleimani, M., Gawenis, L.
   R., Riddle, T. M., Duffy, J. J., Doetschman, T., Wang, T., Giebisch, G., Aronson, P. S.,
   Lorenz, J. N., and Shull, G. E. (1998) Nat Genet 19, 282-285.

5. Grinstein, S., Cohen, S., and Rothstein, A. (1984) J Gen Physiol 83, 341-369.

6. Aronson, P. S., Nee, J., and Suhm, M. A. (1982) Nature 299, 161-163

7. Grinstein, S., Goetz, J. D., and Rothstein, A. (1984) J Gen Physiol 84, 585-600.

8. Kinsella, J. L., Heller, P., and Froehlich, J. P. (1998) Biochem Cell Biol 76, 743-749

9. Shugrue, C. A., Obermuller, N., Bachmann, S., Slayman, C. W., and Reilly, R. F. (1999)
   J Am Soc Nephrol 10, 1649-1657.

10. Rotin, D., and Grinstein, S. (1989) Am J Physiol 257, C1158-C1165

11. Franchi, A., Perucca-Lostanlen, D., and Pouyssegur, J. (1986) Proc Natl Acad Sci USA
    83, 9388-9392

12. Orlowski, J. (1993) J Biol Chem. 268, 16369-16377

13. D’Souza, S., Garcia-Cabado, A., Yu, F., Teter, K., Lukas, G., Skorecki, K., Moore, H.
    P., Orlowski, J., and Grinstein, S. (1998) J. Biol. Chem. 273, 2035-2043

14. Ghosh, S., Gepstein, S., Heikkila, J. J., and Dumbroff, E. B. (1988) Anal Biochem 169,
    227-233.

15. Brown, C. D., Bodmer, M., Biber, J., and Murer, H. (1984) Biochim Biophys Acta 769,
    471-478.
16. Kapus, A., Grinstein, S., Wasan, S., Kandasamy, R., and Orlowski, J. (1994) *J Biol Chem.* **269**, 23544-23552

17. Reilly, R. F., Hildebrandt, F., Biemesderfer, D., Sardet, C., Pouyssegur, J., Aronson, P. S., Slayman, C. W., and Igarashi, P. (1991) *Am J Physiol* **261**, F1088-1094.

18. Orlowski, J., and Kandasamy, R. A. (1996) *J Biol Chem* **271**, 19922-19927

19. Helmle-Kolb, C., Montrose, M. H., and Murer, H. (1990) *Pflugers Arch* **416**, 615-623.

20. Helmle-Kolb, C., Counillon, L., Roux, D., Pouyssegur, J., Mrkic, B., and Murer, H. (1993) *Pflugers Arch* **425**, 34-40.

21. Ishibashi, H., Dinudom, A., Harvey, K. F., Kumar, S., Young, J. A., and Cook, D. I. (1999) *Proc Natl Acad Sci U S A* **96**, 9949-9953.

22. Biemesderfer, D., Rutherford, P. A., Nagy, T., Pizzonia, J. H., Abu-Alfa, A. K., and Aronson, P. S. (1997) *Am J Physiol.* **273**, F289-299

23. Janecki, A. J., Montrose, M. H., Zimniak, P., Zweibaum, A., Tse, C. M., Khurana, S., and Donowitz, M. (1998) *J Biol Chem* **273**, 8790-8798

24. Yang, X., Amemiya, M., Peng, Y., Moe, O. W., Preisig, P. A., and Alpern, R. J. (2000) *Am J Physiol.* **279**, C410-419

25. Kurashima K, S. E., Lukacs G, Orlowski J, Grinstein S. (1998) *J Biol Chem* **273**, 20828-20836

26. Moe, O. W., Amemiya, M., and Yamaji, Y. (1995) *J Clin Invest* **96**, 2187-2194

27. Kurashima, K., Frank, H., Cabado, A. G., Szabo, E. Z., Grinstein, S., and Orlowski, J. (1997) *J. Biol. Chem.* **272**, 28672-28679

28. Wiederkehr, M. R., Zhao, H., and Moe, O. W. (1999) *Am J Physiol* **276**, C1205-1217

29. Zhao, H., Wiederkehr, M. R., Fan, L., Collazo, R. L., Crowder, L. A., and Moe, O. W.
30. Yamaji, Y., Amemiya, M., Cano, A., Preisig, P. A., Miller, R. T., Moe, O. W., and Alpern, R. J. (1995) *Proc Natl Acad Sci U S A* **92**, 6274-6278.

31. Kurashima, K., D'Souza, S., Szaszi, K., Ramjeesingh, R., Orlowski, J., and Grinstein, S. (1999) *J Biol Chem* **274**, 29843-29849.

32. Simons, K., and Ikonen, E. (1997) *Nature* **387**, 569-572.

33. Szabo, E. Z., Numata, M., Shull, G. E., and Orlowski, J. (2000) *J Biol Chem* **275**, 6302-6307.

34. Riegler, M., Sedivy, R., Pothoulakis, C., Hamilton, G., Zacherl, J., Bischof, G., Cosentini, E., Feil, W., Schiessel, R., LaMont, J. T., and et al. (1995) *J Clin Invest* **95**, 2004-2011.

35. Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) *Nature* **375**, 500-503.

36. Horie, S., Moe, O., Tejedor, A., and Alpern, R. J. (1990) *Proc Natl Acad Sci U S A* **87**, 4742-4745.

37. Igarashi, P., Freed, M. I., Ganz, M. B., and Reilly, R. F. (1992) *Am J Physiol* **263**, F83-88.

38. Amemiya, M., Loffing, J., Lotscher, M., Kaissling, B., Alpern, R. J., and Moe, O. W. (1995) *Kidney Int* **48**, 1206-1215.
LEGENDS TO FIGURES

Fig. 1. Effect of varying time of acidification on NHE3 activity in LLC-PK₁ cells.

A: Representative traces of Na⁺-induced pHi recovery. LLC-PK₁ cells were stained with BCECF, incubated with 15 mM NH₄Cl and used for measurement of pHi as detailed under Methods. Sixty sec after initiation of the trace, the cells were transferred to a Na⁺-free solution devoid of NH₄Cl, resulting in a cytosolic acidification. Where indicated by the arrows, Na⁺ was reintroduced. Three different representative traces are superimposed. Amiloride (100 µM) was present throughout to eliminate NHE1 activity. B: Cells were acid-loaded as in (A) and bathed in a K⁺-rich Na⁺-free solution. Where noted by the arrows, 14 µM nigericin was added. Two different representative traces are superimposed. C: Summary of the time dependence of the rate of pHi recovery. Open squares: Na⁺-induced pHi recovery (i.e. NHE activity) calculated from at least 6 experiments like that in (A). Solid squares: nigericin-induced recovery from 5 experiments (1 min) and 3 experiments (5 min) like that in (B). H⁺ fluxes were calculated from the rate of pH₁ change and the cytosolic buffering power, measured independently as described in Methods.

Fig. 2. Effect of intracellular Na⁺ depletion on NHE3 activity. A: Representative traces of Na⁺-induced pHi recovery from acid loading in Na⁺ depleted cells. The cells were pre-incubated with 15 mM NH₄Cl in Na⁺-free solution for 10 min and washed with Na⁺-free solution, to deplete cytosolic Na⁺. Thirty sec after initiation of the trace, the cells were
transferred to a Na\textsuperscript{+}-free solution devoid of NH\textsubscript{4}Cl, resulting in a cytosolic acidification.

Finally, Na\textsuperscript{+} was reintroduced where indicated by the arrows. Two different representative traces are superimposed. **B:** Effect of length of acidification on H\textsuperscript{+} flux in Na\textsuperscript{+} depleted cells. Data are means ± SE of 5 experiments (1 min) and 4 experiments (5 min). * p < 0.05.

**Fig. 3.** Effects of pHi and length of acidification on the activation of NHE3.

**A:** Na\textsuperscript{+}-induced pHi recovery after a 1 min acidification. LLC-PK\textsubscript{1} cells were stained with BCECF. To clamp pHi at the desired level, the cells were incubated with varying concentrations of NH\textsubscript{4}Cl, determined empirically in preliminary experiments. Thirty sec after initiation of the trace, the cells were transferred to a Na\textsuperscript{+}-free solution devoid of NH\textsubscript{4}Cl, resulting in cytosolic acidification. Where indicated by the arrow, Na\textsuperscript{+} was reintroduced. Three different representative traces are superimposed. Amiloride (100 µM) was present throughout to minimize the contribution of NHE1. **B:** Na\textsuperscript{+}-induced pHi recovery after a 5 min acidification. The cells were acidified for 5 min and recovery was induced by reintroduction of Na\textsuperscript{+} (arrow) as in (A). Three different representative traces are superimposed. **C:** pHi dependence of Na\textsuperscript{+}-induced H\textsuperscript{+} (equivalent) efflux following 1 min (open squares) or 5 min (solid squares) acidification periods. H\textsuperscript{+} fluxes were calculated multiplying the rate of pHi change times the cytosolic buffering power, measured independently as described in Methods. The hyperbolic curves were calculated fitting data from 15 and 17 experiments for 1 min and 5 min acidification, respectively, to the
equation Flux = Vmax \cdot \frac{[H^+]}{(K_m + [H^+]}. \ R^2 = 0.781 \ for \ the \ 5 \ min \ curve \ and \ 0.453 \ for \ the \ 1 \ min \ curve.

**Fig. 4.** The surface density and phosphorylation level of NHE3 do not change during acidification.  **A:** Effect of acidification on surface distribution of NHE3.  LLC-PK$_1$ cells stably expressing NHE3’38HA3 were fixed immediately before (control) or after acid loading for 5 min as described under Methods.  NHE3’38HA3 was then immunostained and analyzed by fluorescence microscopy.  Images are representative of 3 experiments.  **B:** Open bars (left axis): quantitation of the number of surface-exposed NHE3, determined in control (left) or acid-loaded cells (right) using $^{125}$I-labeled antibodies.  Data are means ± SE of 9 experiments.  Solid bars (right axis): quantitation of NHE3 phosphorylation in control (left) or acid-loaded cells (right) using $^{32}$P labeling, isolation of brush borders and immunoprecipitation, as detailed in Methods.  Signals were normalized by the amount of NHE3 protein detected by immunoblotting in the same membranes.  Data from 3 separate experiments are expressed as percent of control.

**Fig. 5.** Effect of pH on the interaction of NHE3 with the actin cytoskeleton.  **A:** LLC-PK$_1$ cells stably expressing NHE3’38HA3 were acidified for 5 min and extracted using lysis medium with 0.1% Triton X-100.  The soluble fraction (sup) and insoluble residue (ppt) were analyzed by SDS-PAGE and immunoblotting using anti-HA (top; Mr H 85,000) or anti-actin (bottom; Mr H 45,000).  The immunoblot illustrated is representative of 3 similar experiments.  **B:** Summary of the quantitation of the soluble fraction of NHE3 (solid bars), actin (open bars) and
total protein (hatched bars) following extraction of NHE338HA3-expressing LLC-PK1 cells with lysis solutions containing 0.1% Triton X-100 at pH 6.4 or 7.4, as specified. Data are means ± SE of at least 3 experiments of each type. **C:** Effect of varying time of acidification on NHE3 activity in AP-1 cells stably transfected with NHE3638. Representative traces of Na⁺-induced pHᵢ recovery. The cells were stained with BCECF, incubated with 40 mM NH₄Cl and used for measurement of pHᵢ as detailed under Methods. The cells were transferred to a Na⁺-free solution devoid of NH₄Cl, resulting in a cytosolic acidification. Where indicated by the arrows, Na⁺ was reintroduced. Two different representative traces are superimposed. **D:** Summary of the time dependence of the rate of pHᵢ recovery in NHE3638-transfected cells. To facilitate comparison between experiments, the data were normalized to the recovery rate after 1 min. Means ± SE of 5 determinations. *, p < 0.05.

**Fig. 6.** Rapid inactivation of NHE3 following prolonged acidification. LLC-PK₁ cells were loaded with BCECF and used for pHᵢ determination, as described for Fig. 1. Where indicated, the concentrations of NaCl or NH₄Cl in the perfusate were altered. Note that 6 mM NH₄Cl restore pH to near normal values after acid loading and that subsequent re-introduction of Na⁺ had little effect. Trace is representative of 4 experiments.

**Fig. 7.** Comparison of the effect of varying time of acidification on the activity of NHE isoforms. **A:** Representative traces of Na⁺-induced pHᵢ recovery. CHO cells expressing NHE1 cells were stained with BCECF, incubated with 15 mM NH₄Cl and used for measurement of pHᵢ
as detailed under Methods. Ten sec after initiation of the trace, the cells were transferred to a Na⁺-free solution devoid of NH4Cl, resulting in a cytosolic acidification. Where indicated by the arrows, Na⁺ was reintroduced. Two different representative traces are superimposed. B:

Comparison of the responsiveness of various NHE isoforms to sustained acidification. AP-1 cells transfected stably with the NHE isoform indicated were acid-loaded for either 1 or 3 min and the rate of Na⁺-induced recovery of pHᵢ was measured fluorimetrically, as described in Methods. To facilitate comparison between isoforms, the activity was normalized in each case to that observed after 1 min acidification. Data are means ± SE of at least 4 experiments of each type. *, p < 0.05 compared to 1 min acidification.
A

NHE1

\[ \text{pH}_i \]

Time (sec)

0 100 200 300

B

\[ \text{\% of control} \]

1 min
3 min

NHE1  NHE2  NHE3  NHE5

*
A slow pH-dependent conformational transition underlies a novel mode of activation of the epithelial Na+/H+ exchanger NHE3 isoform

Hisayoshi Hayashi, Katalin Szászi, Natasha Coady-Osberg, John Orlowski, James L. Kinsella and Sergio Grinstein

J. Biol. Chem. published online January 15, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111868200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts