Membrane Curvature Alters the Activation Kinetics of the Epithelial Na\(^+\)/H\(^+\) Exchanger, NHE3\(^{*}\)\(^{5}\)

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The epithelial Na\(^+\)/H\(^+\) exchanger, NHE3, was found to activate slowly following an acute cytosolic acidification. The sigmoidal course of activation could not be explained by the conventional two-state model, which postulates that activation results from protonation of an allosteric modifier site. Instead, mathematical modeling predicted the existence of three distinct states of the exchanger: two different inactive states plus an active form. The interconversion of the inactive states is rapid and dependent on pH, whereas the conversion between the second inactive state and the active conformation is slow and pH-independent but subject to regulation by other stimuli. Accordingly, exposure of epithelial cells to hypoosmolar solutions activated NHE3 by accelerating this latter transition. The number of surface-exposed exchangers and their association with the cytoskeleton were not affected by hypoosmolarity. Instead, NHE3 is activated by the membrane deformation, a result of cell swelling. This was suggested by the stimulatory effects of amphiphiles that induce a comparable positive (convex) deformation of the membrane. We conclude that NHE3 exists in multiple states and that different physiological parameters control the transitions between them.

The activity of sodium-proton exchangers (NHEs) is fundamental to the maintenance of both intracellular and systemic [Na\(^+\)] and pH. Multiple isoforms of NHE have been identified that differ in their tissue distribution and subcellular localization. Some isoforms function primarily in cytosolic cation homeostasis, whereas others are thought to regulate organellar cation transport, accounting for their differential subcellular localization. Similarly, differences in the pattern of expression likely underlie the functional roles of various NHEs; widely expressed isoforms, such as NHE1, have housekeeping activity, whereas those restricted to defined tissues have specialized functions. One such specialized isoform is NHE3, which is expressed almost exclusively on the apical pole of epithelial cells. In the gut and kidney, NHE3 mediates the (re)absorption of salt, bicarbonate, and water (1).

Regardless of their precise function, the activity of all the isoforms studied to date is highly sensitive to the intracellular pH (pH\(_i\)) (2–7). This exquisite pH dependence has been attributed to the protonation of an allosteric site on the cytosolic face of the exchanger (2, 8), which is distinct from the proton transport site (8). According to this two-state model, protonation of the allosteric site converts the exchanger from an inactive to an active form.

In addition to pH\(_i\), some NHE isoforms are sensitive to alterations in osmolarity (9). The best studied example, NHE1, is activated by extracellular hyperosmolarity and inhibited by hypoosmolarity (10–12). The activation of NHE1 induced by hyperosmolarity is felt to be a compensatory response to cell shrinkage, because it causes net salt and water intake, leading to volume restoration. In contrast, hyperosmolarity inhibits epithelial NHE3 activity (12–17). The mechanisms underlying these divergent responses are not clear at present.

The response of NHE3 to hypoosmolarity has been studied less extensively (13, 14). Here we investigated the effect of reduced extracellular osmolarity on NHE3 activity. During the course of these studies, we found that the conventional two-state model of NHE activation was insufficient to account for the behavior of NHE3. We report that an additional inactive state of the exchanger is required to explain its kinetics of activation. The transition between the two inactive (or poorly active) states is pH\(_i\)-independent and limits the rate of activation. Importantly, hypoosmotic stress was found to stimulate NHE3 by accelerating the rate-limiting transition between inactive states. This effect was not caused directly by the hypoosmolarity of the bathing solution but by the curvature imposed on the membrane upon cell swelling.

**EXPERIMENTAL PROCEDURES**

**Materials and Solutions**— Nigericin, the acetoxymethyl ester of 2′,7′-bis(carboxyethyl)-5-(6)-carboxyfluorescein (BCECF),

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\(^{5}\) The on-line version of this article (available at http://www.jbc.org) contains supplemental equations 1–11 and Fig. 1.

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\(^{3}\) The abbreviations used are: BCECF, 2′,7′-bis(carboxyethyl)-5-(6)-carboxyfluorescein (BCECF),

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5-(N-ethyl-N-isopropyl)-amiloride (EIPA), Alexa 488-conjugated goat anti-mouse antibody and F(ab) fragment were obtained from Molecular Probes, Inc. Phosphatidylcholine, lysophosphatidylcholine, and O-phenylenediamine dihydrochloride were from Sigma. Anti-hemagglutinin (HA) mouse antibody and F(ab) fragment were from BabCo. Cy2- and Cy3-chloride were from Sigma. Anti-hemagglutinin (HA) mouse obtained from Molecular Probes, Inc. Phosphatidylcholine, was assessed in MDCK-NHE3 free solution, and Na\(^{+}\)—Methoxymethyl ester and prepulsed with 50 mM NH\(_4\)Cl in HEPES-Tris (pH 7.4). Hypotonic Na\(^{+}\)—rich medium contained 70 mM NaCl, 10 mM N-methylglucammonium chloride, 3 mM KCl, 1 mM MgCl\(_2\), and 20 mM HEPES-Tris (pH 7.4). Isotonic and hypotonic K\(^{+}\)—rich medium had similar composition except that NaCl was replaced by KCl.

**Cells and Constructs**—Madin-Darby canine kidney (MDCK)-II and opossum kidney cells were obtained from ATCC. The MDCK-II cells were stably transfected with NHE3 containing three tandem copies of the influenza virus HA epitope (YPYDVPDYAS) inserted between the first and second membrane-spanning domains, between Arg\(^{58}\) and Phe\(^{59}\) (NHE3\(^{38HA3}\)), generated as described (19). To select a stable line (MDCK-NHE3\(^{38HA3}\)), the cells were cloned by limiting dilution in the presence of 500 μg/ml G418 and screened by immunofluorescence for expression of HA-tagged NHE3. MDCK and opossum kidney cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium with 5% fetal bovine serum in a 5% CO\(_2\) atmosphere. The experiments were performed at least 72 h after the monolayers had reached confluence.

**Measurement of Na\(^{+}\)/H\(^{+}\) Exchange Activity**—NHE3 activity was assessed in MDCK-NHE3\(^{38HA3}\) cells as the rate of Na\(^{+}\)—induced pH\(_{i}\) recovery after an acid load. Dual excitation ratio determinations of the fluorescence of BCECF were used to measure pH\(_{i}\), as previously detailed (12). Briefly, the cells were grown to confluence on 25-mm glass coverslips, placed into Attofluor cell chambers, and mounted on the stage of the microscope. Next, they were loaded with 5 μM BCECF ace-toxymethyl ester and prepulsed with 50 mM NH\(_4\)Cl in HEPES-buffered RPMI at 37 °C for 10 min for subsequent acid loading. Extracellular dye and NH\(_4\)Cl were then washed away with Na\(^{+}\)—free solution, and Na\(^{+}\)/K\(^{+}\) exchange was initiated by reintroduction of Na\(^{+}\) in either the hypotonic or isoosmolar solution. Measurements of NHE3 activity were performed in the presence of 5 μM EIPA to obviate the contribution of NHE1. Intracellular pH (pH\(_{i}\)) was calibrated by equilibrating the cells with K\(^{+}\)—rich medium titrated to defined pH values and containing 10 μg/ml nigericin (20). Calibration was performed immediately after the recovery of pH\(_{i}\) for each experimental condition/osmolarity tested. Fields of cells were therefore not exposed to multiple rounds of acidification and recovery of pH\(_{i}\).

**Fluorescence Recovery after Photobleaching**—The experiments were performed essentially as described previously (21). In brief, MDCK-NHE3\(^{38HA3}\) cells were labeled with mouse anti-HA F(ab) fragment (1:300 dilution in RPMI) and then with secondary Alexa 488-conjugated goat anti-mouse F(ab) fragment (1:500 dilution). The samples placed in Attofluor chambers were mounted on the stage of a confocal laser microscope (Zeiss LSM 510) and bathed in either isoosmolar or hypotonic solution. The apical plane was brought into focus, and two equal areas (2 μm in diameter) were defined. After acquiring two base-line fluorescence measurements, one of the selected areas was irreversibly photobleached, and then the fluorescence of both areas was measured over time. The fractional fluorescence recovery of the bleached area was determined relative to the average of the two prebleach measurements. The unbleached area was used to estimate possible bleaching incurred during image acquisition.

**Kinetic Modeling**—Theoretical models of the potential states of NHE3 activation were generated as detailed in the supplemental material. These models were then used to predict the rate of recovery of pH\(_{i}\) over time. Software developed at the Centre for Computational Biology of the Hospital for Sick Children was used for this purpose.

**NHE3 Quantification**—Determination of surface-exposed and total cellular NHE3 was performed by an immunoperoxidase method, as detailed in Ref. 22. Briefly, to quantify the amount of surface NHE3, the cells were fixed with 4% paraformaldehyde, blocked with 5% donkey serum for 30 min, and incubated with anti-HA epitope antibody (1:1,000 dilution) for 1 h at room temperature. The data are presented as the means ± S.E. of the number of determinations specified.

**RESULTS**

**Hypoosmolarity Activates NHE3**—A line of MDCK-II cells stably expressing an external HA-tagged form of NHE3, characterized previously in Ref. 21 and hereafter referred to as MDCK-NHE3\(^{38HA3}\) was used to study the osmotic regulation of NHE3. The activity of NHE3 in media of varying osmolarity was assessed from measurements of pH\(_{i}\). As illustrated in Fig. 1B, the addition of Na\(^{+}\) to cells that had undergone acid loading by an NH\(_4\)Cl prepulse induced a recovery toward the steady condition/osmolarity tested. Fields of cells were therefore not exposed to multiple rounds of acidification and recovery of pH\(_{i}\).
state pHₐ (≈7.3), which was complete within 400 s under isosmotic conditions. Because MDCK cells express NHE1 on their basolateral membrane, these and all subsequent measurements using MDCK-NHE3<sub>38HA3</sub> cells were performed in the presence of 5 μM EIPA, unless indicated otherwise. EIPA is an isoform-specific inhibitor that, at the concentration used in the Na<sup>+</sup>-containing medium, virtually eliminates NHE1 (and NHE2) activity, without appreciably affecting NHE3 (4, 25). The effect of hypoosmolarity on NHE3 activity was assessed next. Care was taken to impose a comparable acidification and to maintain the concentration of Na<sup>+</sup> constant (70 mM), to make osmolarity the sole variable. Reducing the osmolarity to 200 mosM markedly increased the rate of pH recovery (Fig. 1B). When assessed over the initial 30 s, the rate of Na<sup>+</sup>-induced alkalization was 3-fold greater in hypoosmolar medium, compared with isoosmotic controls (Fig. 1B). Two lines of evidence suggest that the stimulation of transport was attributable to NHE3 and not to incompletely inhibited NHE1. First, we tested the effect of osmolarity on endogenous NHE activity in the parental wild-type MDCK line in the absence of EIPA. As shown in Fig. 1 (A and C), hypoosmolar treatment reduced the rate of pH<sub>i</sub> recovery, which is largely mediated by NHE1 in this case. This was validated by the addition of 5 μM EIPA, which practically eliminated the Na<sup>+</sup>-induced alkalization in these cells (not shown). Second, we also tested the effect of reduced osmolarity in opossum kidney cells, a line known to express NHE3 but not NHE1 (26, 27). Hypoosmolarity also stimulated native NHE3 activity in opossum kidney cells (not illustrated). The rate of proton (equivalent) extrusion is a function of both the change of pH<sub>i</sub> and the buffering power. It was conceivable that, rather than altering the activity of NHE3, hypoosmolarity reduced the buffering power of the cells, accelerating the pH<sub>i</sub> recovery at constant activity of the exchangers. This possibility was discounted by direct measurements of the cellular buffering power under conditions of varying osmolality. In three determinations, the buffering power of MDCK-NHE3<sub>38HA3</sub> cells averaged 10.6 ± 1.6 and 10.2 ± 3.0 mEq/pH unit, respectively, under iso- and hypoosmolar conditions.

**A Two-state Model Cannot Account for the Activation of NHE3**—Perusal of Fig. 1A provides some clues of the mechanism underlying the osmotic activation of NHE3; the course of pH recovery from an acid load is distinctly sigmoidal under isoosmotic conditions, whereas it is hyperbolic following hypoosmolar exposure. The most noticeable effect of hypoosmolar swelling is to obviate the activation lag observed in normal cells. These observations appear to be at odds with the conventional assumption that NHE3 exists in two predominant states; at physiological pH, the exchangers are thought to be in an inactive (or poorly active) unprotonated form, NHE3(I); acidification of the cytosol leads to protonation of the putative “modifier site(s),” converting the exchangers to an active form, NHE3(A) (Fig. 2A), that can effectively exchange Na<sup>+</sup> for H<sup>+</sup> (Fig. 2B). To assess the validity of these assumptions, we used a mathematical model to predict the activity of NHE3 as a function of time and pH. For a simple two-state model the rate of change of pH<sub>i</sub> following an acid load, ΔpH/Δt, is proportional to the fraction of the exchangers in the active configuration and to the concentration of the substrate ions (Fig. 2B, equation 2). The concentration of NHE3(A) is itself dependent on pH<sub>i</sub> and the rate at which it accumulates when pH<sub>i</sub> is altered is dictated by the rate constant of the reversible NHE3(I) to NHE3(A) conversion reaction (Fig. 2B, equation 7). Using these principles, as explained in more detail in the supplemental material, we modeled the time course of pH<sub>i</sub> recovery caused by Na<sup>+</sup>/H<sup>+</sup> exchange following a rapid acidification, such as that accomplished by ammonium removal following a prepulse. These calculations assume that protonation/deprotonation reactions of exposed side chains of membrane proteins are extremely fast (in the submicrosecond range (28)). As is evident from Fig. 2C, the model predicts an initial rate of pH<sub>i</sub> recovery that is proportional to the degree of acidification. Moreover, the slope of the calculated curves is greatest immediately upon acidification. The suitability of the model was tested by comparing these predictions to the experimental results obtained in MDCK cells, under conditions where either NHE1 or NHE3 were the predominant functioning antiporers. As before, the activity of NHE1 was measured in untransfected wild-type cells, whereas NHE3 was assessed in MDCK-NHE3<sub>38HA3</sub> cells in the presence of EIPA. As illustrated in Fig. 2, the predictions made by the model fit well the behavior of NHE1 in MDCK cells, which resembles that reported for this isoform in a variety of other cell types (16, 29). The rate of transport (estimated over 30 s) is highest immediately after acidification (Fig. 2D) and increases with the magnitude of the acid load (Fig. 2D and F). In contrast, because of the activation lag described above, the activity of NHE3 is not greatly affected by pH<sub>i</sub> immediately after acidification (Fig. 2, E and F). Instead, the rate of transport increases gradually, attaining a maximum ≈30–60 s after the pH<sub>i</sub> is changed. Thus, although the two-state model is well suited to explain the behavior of NHE1, it does not predict the sigmoidal response observed for NHE3.
The next level of complexity is illustrated by the existence of a slower, pH-independent transition(s) that follow the step limiting the activation sequence and that one or more slower, pH-independent transition(s) exist. We therefore considered that the rate-limiting transition is pH-insensitive, the initial rate of transport is largely independent of the magnitude of the acid load, as found for NHE3, but not for NHE1 (Fig. 2). We conclude that, when studied under isoosmotic conditions, the characteristic kinetics of activation of NHE3 are best explained by a model consisting of at least three distinct activation states.

Hypoosmolar Activation Accelerates the Rate-limiting Transition between Inactive States—Having developed a model that predicts the behavior of NHE3 under isoosmotic conditions, we proceeded to investigate the means by which hypoosmolarity activates this isoform. As noted above, hypoosmolar challenge appeared to abrogate the lag time normally observed when cells are acid-loaded under isoosmotic conditions (Fig. 1). Because this lag is attributed in the model to the slow conversion between NHE3(I2) and NHE3(A), the effect of reduced osmolarity must be due to an acceleration of this transition. Two predictions can be made on the basis of this interpretation; first, if the conversion is accelerated sufficiently, the initial rate of recovery from an acid load in hypoosmotic medium would become pH-dependent, and second, if measured at longer times after acid loading, the activity of NHE3 would be minimally affected by changes in osmolarity. These predictions were tested in the experiments shown in Fig. 4. In Fig. 4A, the activity of NHE3 was measured immediately after imposition of acid loads of varying magnitude under isoosmotic conditions. Clearly, hypoosmolarity unmasked the pH dependence of the initial rate of recovery, which is not apparent in isoosmotic controls. This differential behavior, however, is abrogated when a sustained intracellular acidification is imposed (accomplished by removal of ammonium using a Na+ -free solution) before the activity of the exchanger is initiated by the addition of extracellular Na+. The time dependence of the response is illustrated in Fig. 4 (B and C). When measured at an identical pH, within the first 2 min of acidification, the activity of NHE3 is greater in hypoosmotic medium than in isoosmotic medium (Fig. 4B). However, when the cells were acid-loaded under isoosmotic conditions, the effect of reduced osmolarity was abolished.

The three-state model depicted in Fig. 3A and B. Each color illustrates the results predicted for a different degree of acid loading. D, time course of pH, recovery mediated by NHE1, measured in wild-type MDCK cells (without EIPA) acidified to different degrees by varying the ammonium prepulse protocol. E, time course of pH, recovery mediated by NHE3, measured in MDCK-NHE3+3HA3 cells in the presence of 5 μM EIPA acidified to different degrees. F, the initial rates of Na+/H+ exchange were calculated from experiments like those in D and E by linear fitting of the initial 30 s of each trace. The results for NHE1 (open circles) and NHE3 (closed squares) are shown as the means of three experiments for each pH in both instances. The modeled data from C are included for comparison (gray triangles).

Kinetic Modeling of NHE3 Activation Is Consistent with a Three-state Model—Our modeling data demonstrate that a two-state model is insufficient to describe the behavior of NHE3 and suggests therefore that additional states of this isoform exist. Specifically, the existence of an activation lag following acid loading implies that protonation of the allosteric site is not the step limiting the activation sequence and that one or more slower, pH-independent transition(s) exist. We therefore tested more complex models to overcome the limitations of the two-state paradigm. The next level of complexity is illustrated in Fig. 3A. This three-state model postulates the existence of two distinct inactive states, NHE3(I1) and NHE3(I2), plus one active state, NHE3(A). The transition between inactive states is considered to be rapid and pH-dependent, and the conversion from NHE3(I2) to NHE(A) the active conformation is slow and rate-limiting.

We proceeded to test the newly proposed model in a similar fashion, by generating recovery curves and comparing them with actual results obtained from experiments in acid-loaded cells. Fig. 3B shows the predicted pH, recovery curves following varying degrees of acid loading, generated using the three-state model. By introducing a slow transition reaction between NHE3(I2) and NHE3(A), the course of alkalization in this case is calculated to be sigmoidal, more closely resembling the behavior of NHE3. Moreover, because the slow transition between NHE3(I2) and NHE3(A) was assumed to be pH-insensitive, the initial rate of transport is largely independent of the magnitude of the acid load, as found for NHE3, but not for NHE1 (Fig. 2). We conclude that, when studied under isoosmotic conditions, the characteristic kinetics of activation of NHE3 are best explained by a model consisting of at least three distinct activation states.
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**FIGURE 3. Application of a three-state model to fit the activation kinetics of NHE3.** A, a diagrammatic representation of a proposed three-state model of NHE3 activation and differential equations derived from this model. The antipporter is proposed to exist in two inactive (or poorly active) unprotonated forms, NHE3(I1) and NHE3(I2), plus one active state, NHE3(A). The transition between inactive and active states is considered to be slow and pH-independent, whereas conversion from NHE3(I1) to NHE3(I2) occurs rapidly through protonation of the modifier site. B, predicted recovery curves based on the three-state model. As in Fig. 2C, each color illustrates the results predicted for a different degree of acid loading. [NHE3(A)] is the concentration of active NHE3, [NHE3(I1)] is the concentration of NHE3 in the first inactive state, and [NHE3(I2)] is the concentration of NHE3 in the second inactive state. k⁺ and k⁻ are the forward and reverse rate constants, respectively, for the conversion between [NHE3(I1)] and [NHE3(I2)], and k⁺ and k⁻ are the forward and reverse rate constants, respectively, for the conversion between [NHE3(I2)] and [NHE3(A)].

**FIGURE 4. Effects of pH and time of acidification on the osmotic response of NHE3.** A, the initial rates of pH change were measured in cells acid-loaded to varying degrees and allowed to undergo recovery in isoosmolar (solid circles) or hypoosmolar medium (open diamonds). The recovery rates were calculated from experiments like those in Fig. 1B by linear fitting of the initial 30 s of each trace. B, MDCK-NHE3/28HA3 cells were acid-loaded, and after 2 min, recovery of pH was induced by reintroduction of Na⁺ in the presence of 5 μM EIPA in either isoosmolar (closed squares) or hypoosmolar medium (open circles). C, MDCK-NHE3/28HA3 cells were acid-loaded, and after 4 min, recovery of pH was induced by reintroduction of Na⁺ in the presence of 5 μM EIPA as in B. The data in B and C are the means ± S.E. of six separate determinations of each type.

were maintained at an acidic pH for a longer period of time (i.e. 4 min), the rate of pH recovery was rapid and not significantly different in both iso- and hypoosmolar media. Note that the sigmoidicity normally observed in isotonic medium was absent in this case, ostensibly because the prolonged acidification allowed the gradual conversion of most of NHE3(I2) to NHE3(A). The time-dependent alteration in the shape of the recovery can be replicated by theoretical curves applying the three-state but not the two-state model (Fig. 2B versus Fig. 3B), validating the use of the more complex paradigm. Importantly, application of the three-state model suggests that hypoosmolar treatment activates NHE3 by accelerating the transition between NHE3(I2) and NHE3(A). This implies that, although the conversion between NHE3(I2) and NHE3(A) is pH-independent, it is sensitive to the osmolarity of the bathing solution or to a consequence thereof, such as changes in cell size or molecular crowding of the cytosol.

Investigation of the Molecular Basis of NHE3 Activation—We next investigated the molecular basis of the volume-induced stimulation of NHE3. This isoform is present not only at the plasma membrane but also in intracellular storage sites, and redistribution between compartments has been suggested as a mechanism to modulate the transport rate (30, 31). We therefore assessed whether reducing the osmolarity altered the surface expression of NHE3. We took advantage of the exofacial location of the HA epitope introduced into NHE3/28HA3 to determine the distribution of exchangers by immunofluorescence microscopy (21, 23). Comparison of intact and permeabilized MDCK-NHE3/28HA3 cells yielded information of the fraction of NHE3 that was surface-exposed following incubation in hypoosmolar or isoosmolar medium. As illustrated in Fig. 5A, there was no striking difference visually in the distribution of NHE3/28HA3 in the two media. A more precise quantitation was made using a colorimetric immunoperoxidase assay. In three similar experiments we found the number of surface-exposed NHE3 to be invariant between isoosmolar and hypoosmolar conditions (Fig. 5B).

Phosphorylation is another means of regulating the activity of NHE isoforms. In the case of NHE3, phosphorylation of Ser552 by protein kinase A is associated with an alteration of exchange activity (32). We used a phospho-specific antibody to assess whether medium osmolarity influences the state of NHE3 phosphorylation. MDCK-NHE3/28HA3 cells exposed to iso- or hypoosmolar medium were solubilized, and the lysates were subjected to SDS-PAGE, followed by immunoblotting with antibodies developed earlier that recognize the phosphorylated form of Ser552 (18). As shown in Fig. 5C, NHE3 underwent an increase in phosphorylation at Ser552 when subjected to hypoosmolarity. Because phosphorylation at Ser552 is known to correlate with a decreased activity of NHE3 (32), the observed phosphorylation cannot account for the stimulation of transport induced by hypoosmolar challenge.

At least two populations of NHE3 are known to exist on the surface of epithelial cells: one that is immobilized through association with the actin cytoskeleton and another one that is mobile in the plane of the membrane (21). The transport activity of these subpopulations might be different, as found for the...
free and megalin-associated forms of NHE3 (33). We therefore analyzed the effects of hypoosmolarity on the association of NHE3 with the cytoskeleton and on its lateral mobility. Cytoskeletal association was gauged biochemically by quantifying the fraction of the exchangers that is insoluble in the nonionic detergent Triton X-100 (1%). The fraction of the total protein extracted in iso- and hypoosmolar solutions was similar (Fig. 6A). More importantly, the detergent-insoluble fraction of NHE3 (approximately 80% under the conditions used) was similarly unaffected (Fig. 6B).

The preceding approach fails to distinguish between the apical and subapical (vesicular) compartments. To more selectively analyze the cytoskeletal association of the subpopulation of NHE3 at the surface membrane, we deduced its lateral mobility by measuring fluorescence recovery after photobleaching. We had reported earlier that mobility measured by fluorescence recovery after photobleaching is a good indicator of the interaction of NHE3 with the actin cytoskeleton (21). The exofacial HA epitopes present in NHE3\(^{38HA3}\) were labeled using a combination of monovalent Fab fragments of antibodies to HA, followed by Alexa 488-conjugated secondary Fab fragments (Fig. 6C). As we reported earlier, under isotonic conditions a large fraction (65–75%) of NHE3 is virtually immobile on the surface of MDCK cells (Fig. 6D). Hypoosmolar treatment did not increase the mobile fraction; in fact, a further decrease in mobility was observed (Fig. 6D). These findings indicate that hypoosmolar swelling did not release NHE3 from its cytoskeletal anchorage and suggest that the immobile form is functionally active.

A Positive Deformation of the Plasma Membrane Reproduces Hypoosmolar Activation of NHE3—Hypoosmolality results in a decrease in mobility was observed (Fig. 6A). More importantly, the detergent-insoluble fraction of NHE3 associated with the cytoskeleton (21). The fluorescence recovery after photobleaching is a good indicator of the interaction of NHE3 with the actin cytoskeleton (21). The exofacial HA epitopes present in NHE3\(^{38HA3}\) were labeled using a combination of monovalent Fab fragments of antibodies to HA, followed by Alexa 488-conjugated secondary Fab fragments (Fig. 6C). As we reported earlier, under isotonic conditions a large fraction (65–75%) of NHE3 is virtually immobile on the surface of MDCK cells (Fig. 6D). Hypoosmolar treatment did not increase the mobile fraction; in fact, a further decrease in mobility was observed (Fig. 6D). These findings indicate that hypoosmolar swelling did not release NHE3 from its cytoskeletal anchorage and suggest that the immobile form is functionally active.

A Positive Deformation of the Plasma Membrane Reproduces Hypoosmolar Activation of NHE3—Hypoosmolality results in cell swelling and a positive deformation of the plasma membrane. Bulging of the apical membrane of MDCK-NHE3 cells under the conditions of our experiments can be appreciated in Fig. 6C. Because membrane-associated proteins can often sense the lateral tension or curvature of the bilayer (34–36), we probed whether NHE3 is responsive to membrane deformation. To this end, we sought a means of inducing deformation that did not involve alteration of the medium osmolality. We attempted to effect a deformation of the plasma membrane under isosmolar conditions by introducing lysophosphatidylcholines (LPC) into its outer leaflet (34, 36). LPC is a cone-shaped, type II lipid that upon insertion into the membrane mimics the effects of cell swelling (Fig. 7A). As a control, we used the cylindrical-shaped phosphatidylcholine (PC). We proceeded to measure NHE3 activity and found that LPC stimulated the exchanger, whereas its cylindrical counterpart, PC, did not (Fig. 7B) and was indistinguishable from cells that were not treated with either LPC or PC (data not shown). The increased rate of pH\(_4\) recovery was not caused by a detergent-like effect of the lysolipid, because alkalinization was observed only after the addition of Na\(^+\). Of note, LPC had a much larger effect on NHE3 activity than on the control experiments, suggesting that LPC increases the activity of NHE3 by other, unrelated means.

NHE3 appears to be sensitive to membrane curvature in a vectorial manner. This was demonstrated using chlorpromazine, an amphipathic drug that induces negative (concave) deformation of the membrane (37). Unlike LPC, which stimulated the exchanger, chlorpromazine had little effect on NHE3 activity (Fig. 7C). Therefore, NHE3 is activated only by positive (convex) deformation of the membrane.

DISCUSSION

The results of our modeling suggest that the conventional two-state model of NHE activation is insufficient to describe the experimental results obtained with NHE3. Specifically the slow, pH-insensitive phase of activation observed immediately after imposition of an acute acid load is not easily reconciled with a simple model where protonation of an allosteric site mediates the transition from the inactive to the active state of the exchanger. Direct measurement found that protonation of exposed protein side chains, including those of membrane proteins, occurs within microseconds (28). Hence, a simple rapid protonation step cannot account for the many seconds required to complete the transition between the inactive and active states of NHE3. Instead, as suggested earlier (38), a much slower conformational change of the protein must be invoked.

Our modeling indicates that the activation lag is best explained by the existence of at least three separate functional states, consistent with the occurrence of a slow conformational
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FIGURE 6. Effect of hypoosmolarity on the cytoskeletal tethering of NHE3. MDCK-NHE3-3HA cells were incubated in isotonic (ISO) or hypotonic (HYPO) medium and subjected to Triton X-100 (Tx) extraction. Samples of the total lysate and of the Triton-soluble and insoluble fractions were analyzed by SDS-PAGE. After transfer to nitrocellulose, equal loading was verified using Fast-Stain (Total protein) and samples were immunoblotted using anti-HA antibodies (IB-HA). A representative blot is shown in A and quantitation of three such blots is summarized in B. In C and D, MDCK-NHE3-3HA cells were surface-labeled with antibodies against the exofacial epitope tag to measure the lateral mobility of NHE3 in the apical membrane by fluorescence recovery after photobleaching. Fab fragments of anti-HA antibodies and Alexa 488-conjugated Fab fragments of anti-mouse (secondary) antibodies were used to avoid cross-linking epitopes on adjacent antiporters. Representative three-dimensional reconstructions of confocal images obtained after incubation in isoosmolar and hypoosmolar solution are shown in C, D, curves illustrating the rate and extent of fluorescence recovery after photobleaching, acquired under isoosmolar (black squares) or hypoosmolar conditions (open circles). The results are the means ± S.E. from 10 separate determinations for each condition.

change following protonation. In Fig. 3A two distinct inactive states, I_1 and I_2, are postulated to interconvert rapidly in a pH-dependent manner; subsequently I_1 undergoes a slow activation to the active state, A. However, one could also envisage an alternative model where a slow pH-insensitive conversion of I_2 to I_1 is followed by a rapid, pH-dependent conformational change that results in activation of transport. Both designs could in principle account for our observations. We chose to favor the former design, because the latter model is more restrictive; it can only fit our data if the forward and reverse rate constants are identical and if the fraction of NHE3 in the I_2 state is negligible. Although possible, these conditions are unlikely. We therefore tentatively favor the more flexible model illustrated in Fig. 3A. Finally, it is noteworthy that although three is the minimum number of states that when applied to the model satisfactorily fits the experimental data, additional states could exist, which may not have been resolved using current methodology.

Unlike NHE3, the behavior of NHE1 can be fitted well by the original two-state model. However, this does not rule out the existence of three states for this isoform. The rapid responsiveness of NHE1 to an acute acidification may suggest that, for NHE1, the basal equilibrium between inactive states may, at rest, be shifted largely to the A form, possibly because of the differential sensitivity of this isoform to cell volume/membrane curvature. Indeed, recent work has demonstrated through mutational analysis that NHE1 can exist in multiple states of activation (39). Much greater temporal resolution, not available with current methods, may be required to elucidate the actual number of states of NHE1.

The three-state model proposed for NHE3 entails the existence of a sizable fraction of inactive exchangers at physiological pH. Although this design may appear inefficient, it confers a large regulatory capacity to the system. Moreover, the presence of a rate-limiting transition between I_2 and A furnishes an additional locus that can be targeted for regulation by parameters other than pH. Accordingly, we find that the stimulatory effects of hypoosmolar medium are best explained by an acceleration of the conversion from I_2 to A. By modulating a different transition, osmolarity can in principle regulate NHE3 in a distinct manner, separately from pH.

How does hypoosmolar stress accelerate the conversion of I_2 to A? Our studies suggest that translocation of exchangers from internal stores to the apical membrane is not implicated. Consequently, the enhanced activity must result from the increased activity of a fixed number of transporters at the apical membrane. The association of NHE3 with the cytoskeleton does not seem to be disrupted by hypoosmolarity, to the extent that neither the lateral mobility nor the detergent solubility of NHE3 appear to be shifted largely to the A form, possibly because of the differential sensitivity of this isoform to cell volume/membrane curvature. Indeed, recent work has demonstrated through mutational analysis that NHE1 can exist in multiple states of activation (39). Much greater temporal resolution, not available with current methods, may be required to elucidate the actual number of states of NHE1.

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hypoosmolar exposure. Changes in plasma membrane curvature may alter the conformation or oligomerization state of NHE3, which could in turn affect its activity, as suggested earlier (40). Alternatively, deformation of the apical membrane might dictate the state of association of NHE3 with other resident plasma membrane proteins, such as calcineurin-homologous protein (41) or phospholipids like phosphatidylinositol 3,4,5-trisphosphate (34) that are known regulators of the exchanger.

As found here for NHE3, curvature changes induced by hydrophobic mismatch were recently reported to alter drastically the activity of NHE1 (34). Of note, LPC and other agents that mimic the effects of cell swelling drastically inhibited NHE1, an effect diametrically opposed to that seen here. However, NHE1 and NHE3 are in fact known to respond in opposite fashion to osmotically induced volume changes; the former is activated by cell shrinkage, whereas the latter is inhibited (12). Therefore, one can envisage a situation where the I1 form of NHE3 predominates at normal membrane curvature and may be favored by concavity, whereas I2 is favored by convex deformation (Fig. 7D), and the reverse may be true for NHE1. One must bear in mind that agents such as LPC and chlorpromazine may cause effects other than deformation of the plasma membrane. These compounds may affect NHE activity indirectly by inducing signaling or though association with ancillary molecules. Measurements of the function of purified NHE in reconstituted membranes may eventually resolve these possibilities.

Interest in the activation of NHE3 by anisoosmolarity is not limited to the laboratory setting or to pathophysiological conditions like those associated with hypoosmolar volume overload (13, 14). Repeated measurements confirm that sodium glucose cotransport activates NHE3 (30, 42). The basis of this activation is not yet clearly defined. However, the rapid influx of glucose and sodium must drive osmotically obliged water into the cells, causing them to swell. We therefore propose that the mechanism for this activation of NHE3 is a perturbation in the membrane curvature induced by cell swelling.

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FIGURE 7. A positive deformation of the plasma membrane reproduces the hypoosmolar activation of NHE3. A, illustration of how cone-shaped amphipathic molecules can alter the curvature of the plasma membrane, depending on their shape and on the monolayer where they insert. B, Na+-dependent, EIPA-insensitive recovery of pH in acid-loaded MDCK-NHE3(-) cells treated with either lysophosphatidylcholine (closed squares) or phosphatidylcholine (open circles). The results are the means ± S.E. of at least six experiments. C, initial rate of NHE3 activity following an acid load in cells treated under isoosmotic conditions with LPC, PC, or chlorpromazine (CPZ) or without additions (Cont). The data are the means ± S.E. of six separate experiments. D, proposed model of NHE3 activation induced by hypoosmolality. Cell swelling is proposed to favor the transition from the first inactive state, NHE3(I1), to the second one NHE3(I2), in a pH-independent manner. As it accumulates, NHE3(I2) can rapidly respond to cytosolic acidification.
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