Endosomal Recycling of the Na\textsuperscript{+}/H\textsuperscript{+} Exchanger NHE3 Isoform Is Regulated by the Phosphatidylinositol 3-Kinase Pathway*  

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The NHE3 isoform of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger localizes to both the plasmalemmal and endosomal compartments in polarized epithelial and transfected Chinese hamster ovary (AP-1) cells. It is unclear how the distribution of NHE3 between these compartments is regulated. In this study, we examined the potential involvement of phosphatidylinositol 3'-kinase (PI3-K) in regulating the activity and distribution of NHE3, as this lipid kinase has been implicated in modulating vesicular traffic in the endosomal recycling pathway. Wortmannin and LY294002, both potent inhibitors of PI3-K, markedly inhibited NHE3-mediated H\textsuperscript{+} extrusion across the plasma membrane in a concentration- and time-dependent manner. The subcellular distribution of the antiporters was monitored by transfecting epitope-tagged NHE3 into AP-1 cells. In parallel with the inhibition of transport, PI3-K antagonists induced a pronounced loss of NHE3 from the cell surface and its accumulation in an intracellular compartment, as assessed by immunofluorescence microscopy and enzyme-linked immunosorbent assays. Further analysis using cells transfected with antiporters bearing an external epitope tag revealed that the redistribution reflected primarily a decrease in the rate of recycling of intracellular NHE3 to the cell surface. The wortmannin-induced inhibition and redistribution of NHE3 were prevented when cells were incubated at 4 °C, consistent with the known temperature dependence of the endocytic process. These observations demonstrate that NHE3 activity is controlled by dynamic endocytic and recycling events that are modulated by PI3-K.

Cellular processes such as pH homeostasis, volume regulation, and transepithelial ion and water transport are mediated, in part, by the active extrusion of cytosolic H\textsuperscript{+} in exchange for extracellular Na\textsuperscript{+} (1–3). This process is electroneutral and mediated by a family of integral membrane Na\textsuperscript{+}/H\textsuperscript{+} antiporters or exchangers (NHE). These transporters may also play a role in cell proliferation (2) and adhesion (4, 5). In mammalian cells, six NHE isoforms (NHE1 to NHE6) have been identified to date (3, 5–9). NHE1 and NHE6 are present in nearly all cells, whereas the other isoforms have a more restricted tissue distribution and fulfill more specialized functions. Among the latter, the best characterized is NHE3, which is confined to certain epithelial cells of the kidney, gastrointestinal tract, and gallbladder, where it participates actively in transepithelial Na\textsuperscript{+} and HCO\textsubscript{3}– absorption. The subcellular distribution of the isoforms also differs. NHE1, the “housekeeping” isoform, is present in the plasmalemma of most cells and accumulates in the basolateral membrane of epithelial cells. The other ubiquitous isoform, NHE6, is found in mitochondria (10). The epithelial isoform NHE3 is detectable in the apical (“brush border”) membrane, and also in subapical vesicles, possibly endosomes (11). Interestingly, this distribution is recapitulated when NHE3 is heterologously expressed in non-polarized cells, such as the antiport-deficient Chinese hamster ovary cell line AP-1 (12). In this case, NHE3 is present and active on the plasma membrane, but is also abundant in a juxtanuclear vesicular compartment. Co-localization with transferrin receptors and with cellubrevin, and dispersal by colchicine indicated that this compartment overlaps with, or is identical to, the recycling endosomes (13). These observations raised the possibility that, as in the case of other transporters, Na\textsuperscript{+}/H\textsuperscript{+} exchange may be regulated in epithelia by recruitment of NHE3 to and from the apical membrane. Indeed, earlier fractionation experiments revealed that the density of the subcellular compartment expressing NHE3 shifted following treatment of renal cells with parathyroid hormone, or after induction of hypertension (14, 15). These findings are consistent with the migration of NHE3 molecules from one intracellular compartment to another in response to physiological stimuli.

Despite these suggestive observations, the mobilization of NHE3 has not been documented directly. Moreover, little is known about the molecular mechanisms regulating the putative translocation process. Some insight may be derived from recent findings made in Caco-2 cells expressing NHE3. These colon carcinoma cells responded to epidermal growth factor with an increase in the rate of Na\textsuperscript{+}/H\textsuperscript{+} exchange, which was eliminated by wortmannin, a potent inhibitor of phosphatidylinositol 3'-kinase (PI3-K). This enzyme catalyzes the phosphorylation of the 3'-position of the inositol ring of phosphoinositides, yielding polyphosphoinositides that seem to play active roles in signal transduction, including the recruitment and activation of several kinases and adaptors to the plasma membrane (see Ref. 16 for review). Importantly, multiple lines of...
evidence have implicated P13-K(s) in vesicular trafficking. The p110 catalytic subunit of the mammalian P13-K is 55% homologous to the Saccharomyces cerevisiae protein VPS-34 (17). Disruption of the VPS-34 gene abolishes P13-K activity in yeast and causes missooring of vacuolar hydrolases to the secretory pathway (18). In mammalian systems, P13-K inhibitors such as wortmannin and LY294002 interfere with membrane traffic in a variety of cell types. They obliterate histamine secretion in RBL-2H3 cells, exocytosis in neutrophils stimulated with chemotactic agents, and translocation of GLUT4 glucose transporters in insulin-stimulated adipocytes (19–25). Finally, mutagenesis of the two P13-K binding sites of the platelet-derived growth factor receptor disrupts the post-endothelial sorting of this receptor (26).

In view of these precedents, we considered the possibility that traffic of NHE3 between the plasmalemmal and endosom-}

| experimental_procedures | materials_and_solutions | Immunofluorescence—AP-1 cells stably expressing HA-tagged NHE3 were plated onto glass coverslips and grown to ~70% confluence. They were washed three times with PBS, and then fixed for 10 min at room temperature using 4% paraformaldehyde in PBS. Following fixation, the cells were washed three to four times with PBS and incubated with 100 μM of the precursor BCECF-acetoxy-ester. Unless otherwise indicated, all incubations were at 37 °C. To acidify the cytosol, the cells were pulsed with 50 mM NH4Cl during the last 15 min of the incubation with BCECF and immediately washed three times with NH4Cl- and NaCl-free solution. Recording of pH were routinely initiated at this point, confirming the effectiveness of the acid load. Washed cells were then incubated with 100 μL of the precursor BCECF-acetoxy-


tinum buffer containing 25 mM HEPES to pH 7.3 and adjusted to 290 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 fluorometric pH measurements contained (in mM): 140 NaCl, 3 KCl, 1 MgCl2, 10 glucose, 20 HEPES, pH 7.3. Isotonic Na+–free medium contained the same salts with the exception of NaCl which was substituted by N-methyl-D-glucamine chloride. Isotonic K+–rich medium had the same composition as Na+–rich medium, except that NaCl was replaced by KCl.

**Construction of Epitope-tagged NHE3**—The wild type rat NHE3 cDNA was previously engineered to contain several unique restriction enzyme sites (21). We then developed a mutation protocol to insert a unique epitope at each of these sites. In order to create a unique epitope tag for each of the constructs, we used the tobacco etch virus HA epitope YPYDVPDYA as the 3′ end of the NHE3 coding region (termed NHE3 Δ3′) using the polymeric chain reaction. The second construct involved engineering a unique NHE3 restriction endonuclease site in the coding region within the first predicted extracellular loop of NHE3, which altered a single amino acid residue at position 38 from Ser to Arg (22,23). A Nor–Nol DNA fragment encoding a triple HA tag in the putative extracellular loop was then inserted into the Nol site (tag inserted between amino acids Arg38 and Phe39) and the modified cDNA/protein was called NHE3 Δ3HA. All constructs were subcloned into the mammalian expression vector pCMV under the control of the enhancer/promoter region of the immediate early gene of human cytomegalovirus. In control experiments, no modifications had no obvious effect on the functional properties of NHE3 when expressed in AP-1 cells. The cDNAs were sequenced to confirm the presence of the mutations and to ensure that other random mutations were not introduced.

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**Quantification of Surface NHE3**—To quantify the amount of surface NHE3, we used an enzyme-linked immunosorbent assay (ELISA). AP-1 cells stably expressing externally tagged NHE3 (NHE3 Δ3HA) were plated onto 12-well plates and grown to ~70% confluence. Next they were incubated with anti-HA antibody (1:1000 dilution) for 1 h at 4 °C, to prevent endocytosis. After washing the cells six times with PBS/0.1% Tween 20, incubation with the anti-HA antibody was then performed for 1 h. Following washing, the plates were incubated in 100 μL of anti-mouse IgG (1:5000 dilution) for 1 h, washed again, and incubated with 100 μL of peroxidase conjugated to anti-mouse IgG for 1 h. The plates were washed again, dried, and incubated in 100 μL of ELISA substrate for 1 h. The absorbance at 405 nm was measured using an automated plate reader. The amount of surface NHE3 was calculated based on the standard curve.
dehydrate in PBS. Following fixation, the cells were washed three to four times with PBS and incubated with 100 mM glycine in PBS for 15 min. Cells were next blocked with 5% donkey serum for 20 min, then incubated with a peroxidase-conjugated donkey anti-mouse antibody (1: 1000 dilution) for 1 h. The cells were washed six more times with PBSαMEM and then incubated with 1 ml of OPD reagent for 15 min at room temperature. The reaction was stopped by adding 250 μl of 3 M HCl. The supernatant was collected and absorbance measured at 492 nm (A_{492}) using a U-2000 spectrophotometer (Hitachi, Tokyo, Japan). In the range studied, A_{492} varied linearly with the amount of peroxidase bound.

RESULTS

Effect of Wortmannin on NHE3 Activity—The potential role of PI3-K in the regulation of NHE3 was tested using wortmannin. This drug rapidly and irreversibly inhibits the mammalian PI3-K by alkylating the catalytic p110 subunit. Wortmannin can effectively inhibit PI3-K in intact cells with an IC_{50} of 5–10 nM (22). AP-1 cells transfected with rat NHE3' HA were incubated with increasing concentrations of wortmannin for 30 min, and Na^+/H^+ exchange activity was subsequently assessed fluorimetrically as the rate of Na^+−induced alkalinization. As shown in Fig. 1A, untreated cells recovered readily from the acid load upon reintroduction of Na^+ (control; open circles). Pretreatment with wortmannin, however, induced a concentration-dependent inhibition of NHE3' HA activity. Similar results were also obtained with the untagged form of NHE3' (data not shown). The similarity of the acidification attained after precycling with ammonium indicates that the inhibitory effect of the drug was not due to alteration of the spectroscopic properties of the pH-sensitive dye or the calibration procedure, or to changes in the buffering capacity of the cells. Accordingly, the rate of ^{22}Na^+ uptake in cells that were maintained at pH 6.3 was similarly inhibited by pretreatment with 100 nM wortmannin, consistent with reduced transport across the cell surface membrane (data not shown). To more precisely evaluate the dose dependence of the inhibitory effect of wortmannin, pH recovery was quantified during the initial 100 s, when the rate of alkalinization is essentially linear at all concentrations. As illustrated in Fig. 1B, half-maximal inhibition was observed around 5 nM, with maximal effects (a ~96% depression of the original rate) being reached at or near 100 nM wortmannin. No further decrease in NHE3' HA activity was observed at concentrations as high as 300 nM. This concentration dependence is consistent with an effect of wortmannin on PI3-K (22). Unless otherwise specified, 100 nM wortmannin was used hereafter.

Effect of LY294002 on NHE3 Activity—To confirm that inactivation of PI3-K is indeed responsible for the inhibition of NHE3, we examined the effect of LY294002, an alternative inhibitor of PI3-K that is chemically unrelated to wortmannin and impairs the enzyme by a different mechanism (31). LY294002, which interacts with the ATP-binding site of the enzyme, inhibits PI3-K in other systems with an IC_{50} in the micromolar range (31). As shown in Fig. 2, pretreatment of the cells with LY294002 greatly depressed NHE3' HA activity. The effect obtained with 50 μM LY294002 is comparable to that of 100 nM wortmannin, confirming the notion that NHE3' HA activity is dependent on PI3-K.

Time Course of the Effect of Wortmannin on NHE3 Activity—Wortmannin is known to enter cells readily and to interact with PI3-K rapidly. Analysis of the time course of inhibition by wortmannin could therefore provide an indication of whether the kinase is directly and continuously required to maintain NHE3' HA activity. As shown in Fig. 3, no inhibition was observed after 5 min and only modest effects were seen at 15 min. The bulk of the inhibition developed between 15 and 30 min and no further effects were seen after 60 min. If, as reported for other systems, inhibition of PI3-K by wortmannin occurs rapidly in CHO cells, the slowly developing inhibition of Na^+/H^+ exchange suggests that one or more intervening steps exist between PI3-K and its effect on NHE3' HA. These could include traffic of NHE3' HA between cellular compartments.

Effect of Wortmannin on the Subcellular Distribution of NHE3—Previous studies revealed that NHE3 is present not only in the plasmalemma, but also in an intracellular vesicular pool that overlaps, at least partly, with early/recycling endo-
Because inhibition of PI3-K has been reported to alter endosomal traffic in some cell types (32, 33), it was conceivable that the effects of wortmannin and LY294002 on NHE3 resulted from an alteration in the partition of exchangers in the plasma membrane. We therefore analyzed the effect of treatment with wortmannin on the subcellular distribution of NHE3\textsuperscript{9HA}, by immunofluorescence detection of the HA epitope attached to its cytoplasmic C terminus. NHE3\textsuperscript{9HA} cells were fixed after varying periods of exposure to wortmannin and permeabilized to gain access to the intracellular epitope of both plasmalemmal and endosomal exchangers. As illustrated in Fig. 4A, and as reported earlier (13), in untreated cells a fraction of NHE3\textsuperscript{9HA} is present diffusely at or near the surface membrane, whereas a sizable fraction accumulates in a juxtanuclear vesicular cluster. A similar distribution was noted 5 min after addition of 100 nM wortmannin (Fig. 4B). By 30 and 60 min (Fig. 4, C and D), however, the fraction of diffuse superficial staining was greatly diminished and the bulk of the fluorescence emanated from the juxtanuclear region. The time course of these events corresponds to the progressive diminution of trans-plasmalemmal Na\textsuperscript{+}/H\textsuperscript{+} exchange activity (Fig. 3), suggesting that inhibition of transport results from internalization of NHE3\textsuperscript{9HA}.

**Temperature Sensitivity of the Inhibition of NHE3 by Wortmannin**—Endocytosis is exquisitely dependent on temperature, being virtually eliminated below 16 °C (35, 36). To define whether internalization mediates the inhibition of NHE3 in wortmannin-treated cells, we analyzed the temperature dependence of the process. The inhibitor was allowed to enter the cells and react with PI3-K for 5 min at 37 °C. Unreacted wortmannin was then washed away and the cells incubated for an additional 60 min at either 4 °C or at 37 °C. The former temperature was chosen to preclude endocytosis during the second incubation. Finally, the cells were perfused with medium prewarmed to 37 °C and the activity and distribution of NHE3\textsuperscript{9HA} were analyzed within 5 min. As shown in Fig. 5A, the inhibition induced by wortmannin persisted in cells incubated at 37 °C, despite the removal of unreacted inhibitor after 5 min. This observation is consistent with inhibition of PI3-K by alkylation, an irreversible reaction (20, 34), and implies that a 5-min incubation with the drug suffices to inhibit PI3-K. Importantly, the sample maintained at 4 °C during the second incubation period was only marginally inhibited (Fig. 5A). This suggests that inhibition of PI3-K must be followed by a secondary, temperature-sensitive event to effectively depress NHE3\textsuperscript{9HA} activity. Endocytosis of surface transporters is likely to be this
to and from endosomes, the ubiquitous isoform NHE1 rate experiments.

After the 60-min incubation period, permeabilized, and subjected to NHE3 finally tagged) and with the putatively externally tagged (Fig. 5, not in those maintained at 4 °C during the second incubation the periphery were observed in the cells incubated at 37 °C, but 4° C (Fig. 5A, B, C) demonstrates that the functional properties of the extracellular punctate compartment of intact NHE3 38HA3 cells implies that: (i) the epitope is indeed extracellular and (ii) that NHE3 recycles continuously from the surface membrane to endosomes. The latter conclusion is supported by the finding that, when treatment with the primary antibody was performed at 4 °C, only diffuse staining at the cell surface was noted (Fig. 8).

The extracellular location of the tag was confirmed by exposing intact cells to anti-HA antibodies. After a 1-h incubation at 37 °C, the cells were washed to remove excess unbound antibody, fixed, permeabilized, and exposed to a secondary Cy3-conjugated antibody. As illustrated in Fig. 7C, this resulted in a punctate staining pattern that strongly resembles that observed in internally tagged NHE3 HA cells exposed to both the primary and secondary antibodies after permeabilization (e.g. Fig. 4A). The ability of extracellular HA antibodies to stain the intracellular punctate compartment of intact NHE3 38HA3 cells implies that: (i) the epitope is indeed extracellular and (ii) that NHE3 recycles continuously from the surface membrane to endosomes. The latter conclusion is supported by the finding that, when treatment with the primary antibody was performed at 4 °C, only diffuse staining at the cell surface was noted (Fig. 8).

Fig. 1D demonstrates that the functional properties of the externally tagged NHE3 resemble those of the parental wild type molecule. As illustrated, cells transfected stably with NHE3 38HA3 recovered effectively from an acute load upon addition of extracellular Na+ (Fig. 7D, open circles). Moreover, pretreatment with wortmannin induced a pronounced inhibition of this response, as reported for the untagged and internally tagged forms of NHE3 (see above).

Wortmannin Decreases the Density of NHE3 at the Cell Surface—NHE3 38HA3 cells were next used to quantify the number of transporters at the cell surface, in order to determine whether wortmannin produces inhibition of transport by reducing the number of NHE3 molecules at the cell surface or their intrinsic activity. The cells were treated with wortmannin for the indicated periods of time, followed by incubation with anti-HA antibody for 1 h at 4 °C. After washing, the cells were fixed and stained with the fluorescent secondary antibody. Because the cells were not permeabilized, only surface exchangers are detected under these conditions, as illustrated in Fig. 8. Of note, secondary step. Accordingly, the wortmannin-induced coalescence of NHE3 HA near the nucleus and its disappearance from the periphery were observed in the cells incubated at 37 °C, but not in those maintained at 4 °C during the second incubation (Fig. 5, B and C).

Effect of Wortmannin on NHE1—Unlike NHE3, which cycles to and from endosomes, the ubiquitous isoform NHE1 HA is almost exclusively located in the plasmalemma (cf. Fig. 6, C and D). If, as postulated, the effect of wortmannin is due to an alteration in the rates of endo- or exocytosis of NHE3, the PI3-K inhibitor should have no effect on NHE1 HA. This prediction was tested experimentally in Fig. 6. As reported above, NHE3 HA was markedly inhibited by pre-treatment with wortmannin (Fig. 6A). By contrast, the PI3-K antagonist had no detectable effect on the activity of NHE1 HA, assessed as the recovery induced by an acid load (Fig. 6B). This finding supports the notion that PI3-K is essential for isoform-specific antiporter traffic between compartments, but not for its catalytic (ion exchange) cycle.

Characterization of Externally Epitope-tagged NHE3 (NHE3 38HA3)—To directly evaluate the fraction of NHE3 present at the cell surface and the rate of internalization and exocytosis, we introduced an epitope tag in the first predicted extracellular loop of NHE3. As shown diagrammatically in Fig. 7A, three HA sequences in tandem were inserted between residues 38 and 39 of rat NHE3 and the resulting construct was transfected into AP-1 cells. A representative immunoblot of cells transfected stably with untagged NHE3, NHE3 HA (internally tagged) and with the putatively externally tagged NHE3 38HA3 are illustrated in Fig. 7B. As reported (35), an immunoreactive band of ~85 kDa was observed in NHE3 HA cells and the specificity of the labeling was confirmed by the absence of staining in the untagged cells. Importantly, the NHE3 38HA3 cells displayed strong immunoreactivity at ~90 kDa. The slightly higher molecular mass of the externally tagged form is attributable to addition of a tandem triple HA tag.
the staining intensity changes little during the first 5 min of treatment with wortmannin, but decreases gradually during the course of the next 30 min, mirroring the decrease in the rate of Na\(^+/\)H\(^+\) exchange (cf. Fig. 3). Although these observations are suggestive of a decrease in the number of exchangers, they are at best qualitative. A more quantitative assessment of the effect of wortmannin on the density of NHE3 at the cell surface was made using secondary antibodies coupled to peroxidase and a spectroscopic immunosorbent assay. The results of six determinations from three independent experiments are presented in Fig. 9. Consistent with the immunofluorescence data, surface NHE3\(^{38HA3}\) molecules were found to decrease progressively during the initial 30 min after addition of wortmannin. The surface density of the exchangers decreased to 28% of the initial level by 30 min, and no further change was observed after 60 min. This decrease can account for most of the inhibition of transport induced by wortmannin.

Effects of Wartmannin on the Rate of Endocytosis of NHE3—The reduction in the density of NHE3 at the cell surface could result from acceleration of the rate of endocytosis, inhibition of recycling or a combination of these events. Modified immunofluorescence and ELISA assays were used to estimate the rates of endo- and exocytosis of NHE3 separately. To measure endocytosis, NHE3\(^{38HA3}\) cells were first incubated with anti-HA antibody for 1 h at 4 °C and washed to remove unbound antibody. After treatment with or without wortmannin for 10 min, the cells were incubated for 20 min at 37 °C to allow internalization of the antiporters and associated antibodies. Next, the cells were returned to 4 °C and incubated with either Cy3- or peroxidase-labeled secondary antibody for 1 h. After additional washing, the fraction of the original NHE3 remaining on the surface was evaluated by fluorescence microscopy or by a colorimetric assay of peroxidase activity, as above. Representative results are illustrated in Fig. 10. The initial amount of NHE3\(^{38HA3}\) labeled at the surface was defined by obviating the incubation at 37 °C. Under these conditions, a substantial amount of diffuse plasmalemmal staining was detected (Fig. 10A), which was defined as 100% in the quantitative peroxi-

Fig. 7. Expression of externally epitope-tagged NHE3 (NHE3\(^{38HA3}\)) in AP-1 cells. A, diagram of the putative topology of NHE3 tagged at position 38 with a tandem triple HA sequence (NHE3\(^{38HA3}\)). The extracellular phase is upward, and the cytosolic domains are shown below the putative 12 helical transmembrane domains. B, immunoblot of extracts of AP-1 cells stably transfected with untagged NHE3, NHE3 tagged at the C terminus with a single HA sequence (NHE3\(^{HA}\)), and NHE3 tagged at position 38 with a triple HA tag (NHE3\(^{38HA3}\)). Equal amounts of protein from whole cell extracts were subjected to SDS-PAGE and blotted onto nitrocellulose filters. The transferred protein was analyzed by immunoblotting with monoclonal anti-HA antibody. B and C are representative of at least three experiments. D, NHE3\(^{38HA3}\) cells were incubated without (open symbols) or with 100 nM wortmannin (solid symbols) for 30 min at 37 °C. Cells were stained with BCECF and acid-loaded as described for Fig. 1. The pH\(_{i}\) recovery induced by the addition of Na\(^+\) was measured fluorimetrically. Results are the means ± S.E. of at least 12 cells from three separate experiments.
statistically significant difference (calculated using two-tailed Student’s t-test). Significance of difference between control and wortmannin was indicated by a single asterisk (*), and original surface.

Double asterisk (**) denotes significant difference and original surface.

Fig. 9. Effect of wortmannin on the surface expression of NHE3: quantitation by ELISA. NHE3<sup>38HA</sup> cells were incubated with 100 nM wortmannin for the indicated time at 37 °C. The cells were then chilled and incubated with monoclonal anti-HA antibody (1:1000 dilution) for 1 h at 4 °C. After washing six times to remove unbound antibody, the cells were fixed with paraformaldehyde and blocked with 5% serum. The cells were then incubated with donkey anti-mouse antibody (1:1000 dilution) at 4 °C for 1 h, then washed six times to remove unbound antibody. After treatment without or with 100 nM wortmannin for the indicated time at 37 °C, the cells were then chilled and blocked with serum. In A-C, the cells were incubated with Cy3-conjugated donkey anti-mouse antibody at 4 °C for 1 h, washed, and observed by fluorescence microscopy. In D, the cells were incubated with donkey anti-mouse antibody conjugated with peroxidase and the activity of peroxidase bound to surface NHE3 was quantified by incubation with OPD substrate and measuring absorbance at 492 nm. Data are means ± S.E. of six determinations from three independent experiments.

Fig. 10. Effect of wortmannin on NHE3 endocytosis. NHE3<sup>38HA</sup> cells were incubated with mouse monoclonal anti-HA antibody (1:1000 dilution) at 4 °C for 1 h, then washed six times to remove unbound antibody. After treatment without or with 100 nM wortmannin for 10 min at 4 °C, the cells were incubated for an additional 20 min at 37 °C to allow endocytosis. The cells were then chilled and blocked with serum. In A-C, the cells were incubated with Cy3-conjugated donkey anti-mouse antibody at 4 °C for 1 h, washed, and observed by fluorescence microscopy. In D, they were incubated with donkey anti-mouse antibody conjugated with peroxidase and the activity of peroxidase bound to surface NHE3 was quantified by ELISA as in Fig. 9. Data are means ± S.E. of eight determinations from four independent experiments. Significance of difference between control and wortmannin was calculated using two-tailed Student’s t-test. Single asterisk (*) denotes statistically significant difference (p < 0.05) between control endocytosis and original surface. Double asterisk (**) denotes significant difference between endocytosis in control and wortmannin-treated cells.

Dose assay (Fig. 10D). After 20 min in the absence of wortmannin, a sizable decrease in the surface staining was noted both microscopically and by quantitation of peroxidase activity (Fig. 10, B and D, respectively). When endocytosis was allowed to proceed after treatment with wortmannin, an even greater fraction of the exofacial NHE3<sup>38HA</sup> was internalized (Figs. 10, C and D). In eight determinations, surface NHE3<sup>38HA</sup> decreased to 18.9 ± 3.6% in wortmannin-treated cells, compared with 28.4 ± 1.4% in the untreated controls. This modest yet significant (p < 0.05) acceleration of the rate of endocytosis could contribute to the net disappearance of surface NHE3 induced by wortmannin.

Exocytosis of NHE3—The method used to estimate the rate of NHE3 recycling is summarized schematically in Fig. 11. Briefly, the NHE3 molecules present at the surface were initially saturated with an excess anti-HA antibody by incubation at 4 °C. Preliminary concentration dependence studies indicated that the concentration of antibody used sufficed to completely ligate the exposed epitopes. After removal and extensive washing of the unbound antibody at 4 °C, internal exchangers were then allowed to reach the surface by incubation at 37 °C for 60 min. The newly exposed exchangers were titrated using a low concentration of anti-HA antibody. The cells were subsequently fixed, permeabilized, and incubated with peroxidase-coupled secondary antibody to quantify the total amount of primary antibody bound. This included the exchangers present initially at the surface, plus those that reached the surface during the 60-min incubation at 37 °C. By subtracting the amount present initially (determined by obliterating the incubation at 37 °C) from the total binding, we were able to obtain a minimum estimate of the amount of internal NHE3 that recycled to the surface during the 60-min period. As shown in the right panel of Fig. 11, treatment with wortmannin severely diminished the ability of NHE3 to recycle to the plasma membrane. The rate of exocytosis decreased to 32.4 ± 1.4% of the control. These results imply that the depletion of surface NHE3 induced by wortmannin is caused largely by the inhibition of exocytosis.

DISCUSSION

NHE3 Undergoes Dynamic Recycling—The present results confirm and extend the earlier observations that suggested that NHE3 resides, in part, in an intracellular recycling compartment. Using an externally tagged construct of NHE3 and comparing intact and permeabilized cells by ELISA, we were able to estimate that nearly 90% of the antiporters are in endomembrane vesicles. By monitoring the disappearance of antibodies bound to the external epitope, we determined that NHE3 cycles dynamically between the plasma membrane and endosomes with a half-life of ~15 min (e.g. Fig. 10). Modulation of this rapid recycling event could provide a means to regulate the density and therefore the activity of the exchangers at the surface membrane. In this context, it is interesting that several conditions that are known to affect the net activity of NHE3, such as changes in pH, osmolarity, or activation of certain protein kinases, are also effective modulators of membrane traffic. The link between these events remains to be established experimentally. However, the results using P13-K antagonists indicate that Na<sup>+</sup>/H<sup>+</sup> exchange across the plasma membrane can in fact be drastically modified by altering the traffic of NHE3 between subcellular compartments.

Mode of Action of Wortmannin—Wortmannin reduced NHE3 activity, most likely by inhibiting P13-K. This is indicated by the irreversible nature of the inhibition, by its concentration dependence (IC<sub>50</sub> in the low nanomolar range) and by the analogous effect of LY249002, a chemically unrelated antagonist of the kinase. Moreover, at concentrations up to 100 nM wortmannin does not inhibit a variety of enzymes that could affect NHE3 activity, including protein kinase A, protein kinase C, myosin light chain kinase, cyclic GMP-dependent pro-

<sup>2</sup> It is conceivable that some of the internalized NHE3 recycled back to the surface during the period studied. In this event, inhibition of exocytosis would appear as enhanced endocytosis.

<sup>3</sup> K. Kurashima, unpublished observations.
tein kinase (36), components of the mitogen-activated protein kinase cascade (37), or S6 kinase (34). Nevertheless, findings based solely on the use of pharmacological agents must be interpreted cautiously, as other lipid kinases can also be inhibited by wortmannin: phosphatidylinositol 4′-kinase is inactivated with half-maximal effects around 100 nm wortmannin (38).

The mode of inhibition of NHE3 by wortmannin appears to be indirect, i.e. with little or no effect on the ability of the antiporter to exchange ions. This was concluded in part from the insensitivity of NHE1, a related antiporter, to the inhibitor. Importantly, the two isoforms share a high degree of homology in their transmembrane domains, which are responsible for ion transport. Instead, it appears more likely that NHE3 activity is reduced by interference with recycling to and from endosomes. Two observations support this conclusion: (i) development of the inhibition is temperature-dependent and (ii) the number of transporters at the surface decreases upon treatment with wortmannin. In fact, the course of depletion of transporters parallels that of inhibition of transport and can quantitatively account for most, though not all of the inhibitory effect: Na+/?H+ exchange is inhibited by nearly 90%, whereas the density of transporters drops by about 70%. The mechanism responsible for the remaining fraction of the inhibition remains obscure.

**Role of PI3-K in NHE3 Internalization and Recycling**—In the transfected AP-1 cells, inhibition of PI3-K was associated with a reduction of NHE3 activity, which reflected a moderate stimulation of its rate of endocytosis and a concomitant, but more substantial, inhibition of its rate of exocytosis. These results parallel recent observations by Martys et al. (39), who found that wortmannin caused a marked reduction of the number of transferrin receptors at the surface of CHO cells, due to the combination of increased internalization and depressed recycling. However, the stimulatory effect of wortmannin on transferrin receptor internalization is by no means universal, although some studies report increased uptake (32, 39), others found no change (33), or even a decreased rate (40). The site of action of PI3-K in the internalization of NHE3 is also undefined. Recent reports suggest that products of the activation of PI3-K enhance binding of adaptins to the membrane, promoting the formation of coated pits (41). It is still unclear, however, whether NHE3 enters the cells via clathrin-coated pits and vesicles.

Although the effects of PI3-K on internalization remain uncertain, the role of this enzyme in exocytosis is firmly established. Wortmannin and/or LY294002 inhibit fusion of endomembranes with the plasma membrane in a variety of systems, including the insulin-stimulated exocytosis of vesicles bearing glucose transporters (19, 24, 42) and secretion of bactericidal granules in neutrophils (43). Importantly, the PI3-K inhibitors also reproducibly block the delivery of recycling endosomes to the plasma membrane (44), as well as fusion of endosomes in vivo and in vitro (40, 45). Conversely, addition of a constitu-tively active form of the catalytic p110 subunit of PI3-K stimulated endosome fusion almost 3-fold, whereas addition of a mutant subunit lacking the kinase domain failed to support fusion (40). Jointly, these findings indicate that PI3-K is an essential component of the machinery supporting endosomal fusion and recycling.

**PI3-K and the Physiological Regulation of NHE3**—Our study suggests that the constitutive activity of PI3-K or a related kinase is important in the maintenance of the steady state level of NHE3 on the plasma membrane, at least in heterologously transfected cells. To the extent that NHE3 has also been localized in endomembrane vesicles in epithelia (11), we anticipate that similar effects will be found in native systems. In addition, it is possible that the known effects of some growth promoters on NHE3 may also be mediated by PI3-K dependent vesicular traffic. Specifically, epidermal growth factor was found to stimulate NHE3 activity and this activation was blocked by wortmannin (46). However, caution must be exercised in interpreting these observations as indicative of changes in vesicular traffic, as similar observations have been made in cells expressing NHE1. Ma et al. (47) reported that PI3-K is involved in the activation of NHE1-mediated Na+/?H+ exchange by platele-t-derived growth factor. Because NHE1 resides almost entirely in the plasma membrane of both quiescent and activated cells, the stimulatory effect of PI3-K cannot be mediated by recruitment of endomembrane transporters. Therefore, it is apparent that further studies are required to define the contribution of membrane traffic to the regulation of NHE3, as well as all the sites of action of the products of PI3-K.

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