Activation of Na\textsuperscript{+}/H\textsuperscript{+} Exchange Is Required for Regulatory Volume Decrease after Modest “Physiological” Volume Increases in Jejunal Villus Epithelial Cells*

R. John MacLeod‡ and J. Richard Hamilton

From the Department of Pediatrics, McGill University, Montreal Children’s Hospital Research Institute, Montreal, Quebec H3H 1P3, Canada

Epithelial cell volume increases that occur because of the uptake of Na\textsuperscript{+}-cotransported solutes or hypotonic dilution are followed by a regulatory volume decrease (RVD) due to the activation of K\textsuperscript{+} and Cl\textsuperscript{−} channels. We studied the relationship of Na\textsuperscript{+}/H\textsuperscript{+} exchange (NHE) to this RVD in suspended guinea pig jejunal villus cells, using electronic sizing to measure cell volume changes and fluorescent spectroscopy of cells loaded with 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein to monitor intracellular pH (pH\textsubscript{i}). When the volume increase achieved by these cells during Na\textsuperscript{+} solute absorption was duplicated by a modest 5–7% hypotonic dilution, their pH\textsubscript{i} first acidified and then alkalinized. This alkalization was blocked by 5-(N-methyl-N-isobutyl) amiloride (MIA; 1 μM), an inhibitor of NHE. The RVD subsequent to 5–7% hypotonic dilution was prevented by Na\textsuperscript{+}-free medium and by amiloride and non-amiloride derivatives. The order of potency of these inhibitors was as follows: MIA > 5-(N,N-dimethyl) amiloride > cimetidine > clonidine, in keeping with the pattern attributable to NHE-1 as the isoform of NHE responsible for increase in pH\textsubscript{i} after modest volume increases. A substantial 30% hypotonic dilution caused acidification, and RVD following this larger volume increase was not affected by MIA. To assess the effect of hypotonicity on the activity of NHE, we measured the rate of MIA-sensitive pH\textsubscript{i} recovery from an acid load (dPH\textsubscript{i}/dt) in 5 and 30% hypotonic media. pH\textsubscript{i} recovery was faster in 5% hypotonic medium compared with isotonic medium and slowest in 30% hypotonic medium, which suggested that the activity of NHE was stimulated in the slightly hypotonic medium, but inhibited in the 30% hypotonic medium. To determine the role of activated NHE in RVD after a modest volume increase, cells were hypotonically diluted 7% in MIA to prevent RVD and then alkalinized by NH\textsubscript{4}Cl or acidified by propionic acid addition. Only after alkalinization was there complete volume regulation. We conclude that in Na\textsuperscript{+}-absorbing enterocytes, the NHE-1 isoform of Na\textsuperscript{+}/H\textsuperscript{+} exchange is stimulated by volume increases that duplicate the “physiological” volume increase occurring when these cells absorb Na\textsuperscript{+}-cotransported solutes. The subsequent alkalinization of pH\textsubscript{i} is a required determinant of the osmolyte loss that underlies this distinct volume regulatory mechanism.

*A modest 5–7% volume increase in a jejunal villus epithelial cell is of interest because it duplicates the size to which these cells swell during absorption of Na\textsuperscript{+} solute (1–3). With any such volume increase, these cells activate K\textsuperscript{+} and Cl\textsuperscript{−} channels, causing a regulatory volume decrease (RVD); the resultant KCl efflux returns the volume to normal (4, 5). Because reports using symmetrical mammalian cells have suggested that intracellular pH (pHi) is a determinant of volume regulation (6–8), our efforts to characterize RVD after modest 5–7% swelling focused on pHi, and its relationship to Na\textsuperscript{+}/H\textsuperscript{+} exchange (NHE) activity as cell volume was increased experimentally. In this report, using suspended jejunal villus epithelial cells exposed to a slight hypotonic challenge (0.95 × isotonic dilution) to duplicate the volume increase occurring because of Na\textsuperscript{+} solute absorption (5–7% swelling), we show a precise relationship between NHE activity, pHi, and the activation of ion channels for RVD. This sequence of events differs greatly from that observed after a “standard” hypotonic challenge (0.70 to 0.50 × isotonic dilution), where after cell swelling of 15–20%, NHE activity is inhibited (9–11).

NHE has been identified in many cell types (12, 13), and four distinct isoforms have been cloned (14–18). We have characterized NHE activation and its relationship to RVD by measuring changes in cell volume, pHi, Na\textsuperscript{+} influx, and rates of pHi recovery from an acid load. By showing distinct differences in the response to cell swelling of different magnitudes, we have provided new insight into the mechanism of signal transduction for volume regulation in absorptive epithelial cells.

MATERIALS AND METHODS

Solutions and Reagents—Volume measurements were made on cells suspended in Na\textsuperscript{+} medium at a density of 30,000 cells/ml. This medium contained 140 mM NaCl, 3 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM Na-gluconate, and 10 mM Hepes (pH 7.3; 295 mosm). Na\textsuperscript{+}-free medium and K\textsuperscript{+}-rich medium were made by iso-osmotic replacement of NaCl with the chloride salts of N-methyl-D-glucamine and K\textsuperscript{+}, respectively, and titrated to pH 7.3 with the corresponding bases. Isotonic low Na\textsuperscript{+} medium contained 25 mM NaCl with 115 mM N-methyl-D-glucamine and was used in all pH\textsubscript{i} recovery from ammonium prepulse experiments. Hypotonic solutions (5 and 30%) were made by an appropriate addition of distilled water. Na\textsuperscript{+} uptake buffer was Na\textsuperscript{+} medium supplemented with bovine serum albumin (type V) at 1 mg/ml.

We purchased bafilomycin A1 from Dr. K. Altendorf (Universität Osnabrück, Osnabrück, Germany). The acetoxymethyl ester of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein was obtained from Molecular Probes, Inc. (Eugene, OR). 5-(N-Methyl-N-isobutyl) amiloride (MIA) and 5-(N,N-dimethyl) amiloride were from Research Biochemicals Inc. (Natick, MA), and N-methyl-D-glucamine was from Aldrich. Nigericin, cimetidine, and clonidine were from Sigma, and RPMI 1640 medium (10×) was from Life Technologies, Inc.). Dinonyl phthalate was from...
Pfaltz and Bauer Inc. (Waterbury, CT), and 22NaCl was purchased from Amersham (Montreal, Quebec).

**Villus Cell Isolation and Volume Determination**—Villus cells were isolated from segments of adult male (200–300 g) guinea pig jejunum by mechanical vibration as described previously (19). We resuspended isolated cells at 0.8–1.0 × 10^6 cells/ml in RPMI 1640 medium (without HCO_3\(^{-}\)) containing 1 mg/ml bovine serum albumin (type V) and 20 mM NaHepes (pH 7.3) at 37°C. Three hours after suspension in medium, viability was assessed by trypan blue exclusion. Cell volume was measured using a Coulter Counter (model ZM) with an attached Channelizer (C-256) as described previously (1, 2, 4). Villus cell volume measured electronically over a range of tonicities correlated (r = 0.9867) with direct measurements of cell water (4). The effect of amiloride and non-amiloride inhibitors illustrated in Fig. 8 was measured using an attenuation setting of 32. We determined relative cell volume as the ratio of cell volume under study conditions to the volume under basal conditions in isotonic medium.

**pH\(_{i}\) Measurement and Manipulation**—For the fluorometric determination of pH\(_{i}\), villus cell suspensions (1 × 10^6 cells/ml) in Hepes/RPMI 1640 medium) were loaded with 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein by incubation with the parent acetoxymethyl ester (3.7 μM) for 15 min at 37°C. After washing, 0.5–0.8 × 10^6 cells were used for fluorescence determination in 2 ml of the indicated medium using a Hitachi F-4000 fluorometer with excitation at 495 nm and emission at 525 nm using 5- and 10-nm slits, respectively. We acid-loaded cells by preincubating 10^6 cells/ml in Hepes/RPMI medium containing 2.5 mM NH_4Cl at 37°C, followed by sedimentation and resuspension in 2 ml of the indicated NH_4\(^{+}\)-free medium. For experiments using acid-loaded cells, loading with NH_4\(^{+}\) and 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein was performed simultaneously as described (20). The MIA-sensitive rate of pH\(_{i}\) recovery was the difference between pH\(_{i}\) recovery in the presence of bafilomycin (100 nm) and Zn\(^{2+}\) (100 μM) and pH\(_{i}\) recovery in the presence of these inhibitors and 1 μM MIA. Rates of pH\(_{i}\) recovery were determined in low Na\(^{+}\) medium (25 mM) as described above. Calibration was performed in K\(^{+}\)-medium with an attached nigericin (21) using a quench correction factor as described (22).

**Uptake of 22Na**—We measured the initial rate of 22Na influx with a modified version of a procedure previously described (19). Each villus cell preparation was divided in half and resuspended at a final concentration of 5–6 mg of protein/ml in prewarmed uptake medium in a continuously stirred cuvette. This medium contained 10% bovine serum albumin to inhibit NaK-ATPase cotransport. Uptake was initiated by the addition of 22Na at a concentration of 5–10 μCi/ml. Immediately afterwards, a 500-μl cell suspension was removed and added to 500 μl of ice-cold 1.0 M MgCl\(_{2}\). This aliquot, which took <5 s to obtain, was taken to represent extracellular 22Na associated with the cell pellet. Uptake was terminated after 90 s by diluting 500 μl of cell suspension in an equal volume of ice-cold 0.1 M MgCl\(_{2}\) which was then gently layered on a 100-μl layer of di-n-butyl phthalate-di-n-nonyl phthalate (3:2, v/v) and centrifuged in 2 ml of the indicated medium using a Hitachi F-4000 fluorometer with excitation at 495 nm and emission at 525 nm using 5- and 10-nm slits, respectively.

**Effect of 0.95 × isotonic or 0.70 × isotonic dilution on pH\(_{i}\) with Villus Cell Swelling**—The resting pH\(_{i}\) of villus cells was measured using a Coulter Counter (model ZM) with an attached Channelizer (C-256) as described previously (1, 2, 4). Villus cell volume measured electronically over a range of tonicities correlated (r = 0.9867) with direct measurements of cell water (4). The effect of amiloride and non-amiloride inhibitors illustrated in Fig. 8 was measured using an attenuation setting of 32. We determined relative cell volume as the ratio of cell volume under study conditions to the volume under basal conditions in isotonic medium.

**Statistics**—Data are reported as means ± S.E. of 5 to 16 experiments performed in duplicate. Differences in means were determined using Student’s t test.

**RESULTS**

**Changes in pH\(_{i}\) with Villus Cell Swelling**—The resting pH\(_{i}\) of villus cells in Hepes-buffered RPMI 1640 medium (nominally HCO\(_{3}\)-free) was 7.39 ± 0.04 (n = 45). Fig. 1 illustrates the changes in pH\(_{i}\) of villus cells in suspension hypotonically diluted 5 or 30%. To mimic the volume increase that occurs because of the uptake of either L-alanine or D-glucose (2, 3), the villus cells were diluted 0.95 × isotonic (Fig. 1A). This dilution, which generated a modest volume increase and caused the pH\(_{i}\) to acidify 0.03 ± 0.01 pH units (n = 16), was followed by alkalization. This alkalization after 0.95 × isotonic dilution was prevented by 1 μM MIA (Fig. 1B). After 0.70 × isotonic dilution, which generated a substantial volume increase, the cells acidified 0.105 ± 0.041 pH units (n = 16) (Fig. 1C). Unlike cells suspended at 0.95 × isotonic dilution, these cells continued to acidify over 3 min, and MIA (1 μM) increased this acidification (Fig. 1D). These pH\(_{i}\) changes are summarized in Fig. 2. 0.95 × isotonic dilution caused a ΔpH/3 min of 0.070 ± 0.010 pH units, which was abolished by 1 μM MIA (0.020 ± 0.10 pH units; p < 0.005). Similarly, 0.93 × basal dilution caused MIA-sensitive alkalization (ΔpH\(_{i}\) = 0.050 ± 0.010 versus 0.01 ± 0.01, n = 16; p < 0.001). In contrast, 0.70 × isotonic dilution caused acidification that was increased by MIA (1 μM) (ΔpH\(_{i}\) = −0.016 ± 0.005 versus −0.030 ± 0.005, n = 16; p < 0.005).

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The recovery of pH\(_{i}\) was determined after 0.95 × isotonic dilution was prevented by 1 μM MIA (final relative volume = 1.03 ± 0.01, n = 6; p < 0.001). When we replaced all medium Na\(^{+}\) isotonically with N-methyl-D-glucamine, RVD following 0.95 × isotonic dilution was prevented (final relative volume = 1.04 ± 0.01, n = 6; p < 0.001) (Fig. 3B). RVD after 0.93 × isotonic dilution was also prevented by 1 μM MIA (final relative volume = 1.05 ± 0.01, n = 6; p < 0.001) (Fig. 3C). RVD after 0.93 × isotonic dilution was also prevented in Na\(^{+}\)-free medium (final relative volume = 1.05 ± 0.01, n = 6; p < 0.001) (Fig. 3D).

The effect of Na\(^{+}\)-free medium on RVD following greater volume increases occurring in a very hypotonic medium is illustrated in Fig. 4. After 0.7 × isotonic dilution or 0.8 × isotonic dilution, the subsequent RVD was complete. Consistent with these findings, MIA (1 μM) had no effect on RVD of villus cells after 0.7 × isotonic dilution in Na\(^{+}\)-containing medium (extent of volume decrease = 19 ± 1 versus 19 ± 1%, n =...
MIA (1 μM) in all cases where RVD was inhibited (p < 0.001). As illustrated in Fig. 5, villus cells acidified to a pH of ~6.95 by ammonium prepulse exhibit recovery. The addition of 2.5 mM NH₄Cl to these cells caused an alkalinization of 0.15 ± 0.02 pH units (n = 4) (Fig. 5A). The pH then declined (0.09 ± 0.02 pH units) over the next 5 min and did not change thereafter. When these cells were resuspended in isotonic medium (NH₄Cl-free), the time course of recovery was first-order for 5 min and was ~41% complete in 10 min (Fig. 5B). When cells were suspended in 0.95 × isotonic medium, they recovered their pH with a time course that was first-order for 5 min and ~57% complete in 10 min (Fig. 5C). The rate of pH recovery measured over the first 5 min was faster in 0.95 × isotonic medium than in isotonic medium (dpH/dt × 10⁻² pH units/min = 3.48 ± 0.27 versus 1.59 ± 0.25, n = 6; p < 0.001).

We then assessed the effect of MIA on the rate of pH recovery. In isotonic medium (Fig. 5D), MIA slightly diminished pH recovery (Fig. 5E). In 0.95 × isotonic medium, MIA substantially diminished pH recovery (Fig. 5, F and G). The MIA-sensitive rate of pH recovery was greater in 0.95 × isotonic medium compared with isotonic medium (1.18 ± 0.15 versus 0.43 ± 0.03) × 10⁻² pH units/min, n = 5; p < 0.001). Because the MIA-sensitive rate of pH recovery in isotonic medium was low, we speculated that identifying and controlling amiloride-insensitive sources of pH recovery would better resolve the MIA-sensitive component of pH recovery from an acid load in these cells.

The effects on pH recovery following ammonium prepulse of bafilomycin A₁, a potent and selective inhibitor of type V H⁺-ATPase, and of Zn²⁺, an inhibitor of H⁺ conductance, are illustrated in Fig. 6. The initial rate of pH recovery was first-order and 15% complete (Fig. 6, A). Bafilomycin (100 nm) reduced the extent of pH recovery (ΔpH/2 min = 64.1 ± 9.1%; p < 0.005) (Fig. 6, A and C). The inclusion of Zn²⁺ (100 μM) in the presence of bafilomycin further diminished the extent of pH recovery (ΔpH/2 min = 25.4 ± 2.9%; p < 0.02) (Fig. 6, B and C). These results suggest that ~75% of pH recovery in isotonic medium is amiloride-insensitive.

**Effect of Hypotonicity on Initial Rate of MIA-sensitive pH Recovery from Intracellular Acidification**—To determine the effect of hypotonicity on the activity of NHE, we measured the initial rate of MIA (1 μM)-sensitive pH recovery following ammonium prepulse in the presence of bafilomycin and Zn²⁺ in 0.95 × isotonic medium, MIA slightly diminished pH recovery (Fig. 5E). In 0.95 × isotonic medium, MIA substantially diminished pH recovery (Fig. 5, F and G). The MIA-sensitive rate of pH recovery was greater in 0.95 × isotonic medium compared with isotonic medium (1.18 ± 0.15 versus 0.43 ± 0.03) × 10⁻² pH units/min, n = 5; p < 0.001). Because the MIA-sensitive rate of pH recovery in isotonic medium was low, we speculated that identifying and controlling amiloride-insensitive sources of pH recovery would better resolve the MIA-sensitive component of pH recovery from an acid load in these cells.

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media of different tonicities (Fig. 7). In isotonic media, MIA (1 μM) had only a slight effect on pH recovery (Fig. 7A), but in 0.95 × isotonic medium, MIA completely blocked pH recovery (Fig. 7B). In 0.70 × isotonic medium, the villus cells continued to acidify; MIA attenuated this acidification (Fig. 7C). The initial rates of MIA-sensitive pH recovery are summarized in Table I. In all cases, the starting pH was the same. In isotonic medium, the initial rate of MIA-sensitive pH recovery was (1.16 ± 0.23) × 10⁻² pH units/min. This rate was substantially increased in 0.95 × isotonic medium (3.25 ± 0.26) × 10⁻² pH units/min; p < 0.001). In 0.70 × isotonic medium, the rate was inhibited (0.31 ± 0.20) × 10⁻² pH units/min; p < 0.02). Clearly, the activation of MIA-sensitive Na⁺/H⁺ exchange by 5% hypotonic swelling was not due to intracellular acidification.

**Determination of NHE Isoform Activated by 5% Hypotonicity**—We measured the relative pharmacological sensitivities of RVD after 0.95 × isotonic dilution to several NHE inhibitors, both amiloride and non-amiloride derivatives. Cimetidine (25 μM) attenuated the rate of RVD in comparison with clomidine (50 μM) (Fig. 8A). The relative volume of cells treated with cimetidine at 5 min was greater than that of cells treated with clomidine (relative volume = 1.02 ± 0.01 versus 1.00, n = 7; p < 0.05). Concentration-response profiles for inhibition of RVD after 0.95 × isotonic dilution are illustrated in Fig. 8B. Cimetidine was six times more potent than clomidine. The EC₅₀ values of cimetidine (20 μM) and clomidine (130 μM) were greater than those of 5-((N,N-dimethyl) amiloride (1 μM) and 5-((N-methyl-N-isobutyl) amiloride (220 mM). The order of potency of these inhibitors of the isoform of NHE activated by the 5% volume increase was as follows: MIA > 5-((N,N-dimethyl) amiloride > cimetidine > clomidine.

To determine the effect of extracellular K⁺ on the isoform of NHE activated by 5% hypotonicity, we measured the change in pH of the villus cells suspended in isotonic K⁺-rich medium. After 0.95 × isotonic dilution, the increase in pH was substantially diminished in 140 mM K⁺ compared with 3 mM K⁺ (ΔpH/3 min = 0.020 ± 0.010 versus 0.070 ± 0.010 pH units, n = 6; p < 0.001).

**NH₄Cl-induced Alkalization Allows RVD When NHE Is Inhibited**—Since increasing osmolyte influx (Na⁺) when the villus cells are losing K⁺ and Cl⁻ for RVD seems counterintuitive, we measured the volume and ΔpH in cells hypotonically diluted 7% in the presence of MIA (1 μM) and following the addition of 1 mM NH₄Cl (Fig. 9). As previously observed, MIA (1 μM) prevented RVD after cell swelling following 7% hypotonic dilution (Fig. 9A). The addition of 1 mM NH₄Cl to the swollen cells caused RVD in the presence of MIA. Within 2 min of the addition of NH₄Cl, these cells started to shrink, and RVD was complete in the next 3 min (relative volume = 1.03 ± 0.01 versus 1.00 ± 0.01; p < 0.001). Immediately after the addition of 1 mM NH₄Cl, the villus cells alkalized (Fig. 9B). This alkalization (0.086 ± 0.010 pH units, n = 15) was no different than that measured in these cells following 5% hypotonic dilution (Fig. 2). We then acidified the pH, of comparably treated cells to show the converse of the alkalization experiment (Fig. 9, C and D). Sodium propionate (2 mM) added to cells hypotonically diluted 7% in the presence of MIA had no effect on the
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FIG. 7. MIA-sensitive pH$_i$ recovery from acid load in media of different tonicities. A, isotonic medium containing bafilomycin and Zn$^{2+}$ compared with MIA (1 µM), bafilomycin, and Zn$^{2+}$; B, 0.95 × isotonic medium, bafilomycin, and Zn$^{2+}$ compared with MIA (1 µM), bafilomycin, and Zn$^{2+}$; C, 0.70 × isotonic medium, bafilomycin, and Zn$^{2+}$ compared with MIA, bafilomycin, and Zn$^{2+}$. Results of a single experiment are illustrated.

**Table 1**

|  | Bafilomycin | Bafilomycin | MIA-sensitive |
|---|---|---|---|
| Isotonic ($n = 7$), pH$_i$ | 6.90 ± 0.03 | 6.84 ± 0.03 | 6.90 ± 0.03 |
| 5% hypotonic ($n = 8$), pH$_i$ | 6.92 ± 0.01 | 6.92 ± 0.01 | 6.89 ± 0.03 |
| 30% hypotonic ($n = 14$), pH$_i$ | 6.93 ± 0.03 | 6.93 ± 0.03 | 6.89 ± 0.03 |

a $p < 0.001$ versus isotonic.
b $p < 0.02$ versus isotonic.

inhibited RVD (Fig. 9C). The addition of sodium propionate to these cells caused an acidification (0.086 ± 0.016 pH units, $n = 10$) that remained stable for the next 5 min of the experiment (Fig. 9D). This experiment suggests that it is alkalization of pH$_i$ caused by the NHE activated by modest swelling that signals the ion conductances for the subsequent volume regulation.

**DISCUSSION**

Our results indicate that a modest cell volume increase of 5–7% activates NHE, while an increase of 15% caused by standard hypotonic dilution inhibits NHE. Furthermore, RVD following the modest volume increase of 5–7% absolutely requires activated NHE. We base this interpretation of our results on experiments that isolated the function of NHE during modest volume increases to show amiloride-sensitive alkalization of pH$_i$ and increases in both $^{22}$Na influx and pH$_i$ recovery from an acid load in slightly hypotonic (0.95 × isotonic) medium. Evidence that activated NHE was required for this RVD came from experiments showing that extracellular Na$^+$ was required for the subsequent cell shrinkage and inhibitor sensitivity of RVD. Our results also indicate that it is the alkalization of pH$_i$ from activated NHE that is required for RVD after modest volume increases as transient alkalization of pH$_i$ caused cell shrinkage when an amiloride derivative had prevented volume regulation.

RVD following a modest volume increase was prevented by the non-amiloride derivatives cimetidine and clonidine, with cimetidine being six times more potent than clonidine. This observation provides strong evidence that NHE-1 is the isoform activated by the 5–7% volume increase. Studies using transfectants of NHE isoforms have shown that clonidine is more potent than cimetidine in inhibiting NHE-2 and NHE-3, while only with NHE-1 is this order of potency reversed (25, 26). K$^+$, a weak competitive inhibitor of NHE-1, but not of NHE-2 or NHE-3 (26), prevented the alkalization of pH$_i$ stimulated by the slight (0.95 × isotonic) hypotonic dilution, supporting the interpretation that the isoform responsible for these pH$_i$ effects is NHE-1. Jejunal villus epithelial cells possess three isoforms of NHE (17, 27, 28). NHE-3 and NHE-2 are found on the apical membrane and are both more sensitive to clonidine than to cimetidine, while NHE-1, which is more sensitive to cimetidine than to clonidine, has been localized to the basolateral membrane of villus cells (27–29). We found that MIA was more potent than dimethyl amiloride in preventing RVD after the modest 5–7% volume increase, but this hierarchy is the same for NHE-1, -2, and -3 (26). After a 5% volume increase, but not a 15% volume increase, RVD, the alkalization of pH$_i$, the increased $^{22}$Na influx, and recovery of pH$_i$ from an acid load were all prevented by a low concentration of the N-5-alkyl amiloride derivative. As cimetidine was more potent than clonidine in preventing this RVD, when taken together, our data strongly suggest that NHE-1 is the isoform of NHE activated during the modest 5–7% volume increase in the villus cells.

The fact that the MIA-sensitive rate of pH$_i$ recovery from an acid load was accelerated in cells suspended in 0.95 × isotonic medium but inhibited in cells suspended in the standard hypotonic (0.70 × isotonic) medium suggests that intracellular acidification is not directly related to activation of NHE-1. The well documented kinetic asymmetry of NHE-1 in symmetrical conditions (12), evidenced by a sigmoidal relationship between the Na$^+$/H$^+$ exchange rate and internal H$^+$ concentration, suggests that such cooperativity is because of an intracellular H$^+$ modifier site, distinct from the H$^+$ transport site, and that regulation of NHE occurs via changes in the affinity of this internal H$^+$ modifier site for intracellular H$^+$ (12, 30). We measured differences in the rate of pH$_i$ recovery from an acid load after resuspending the villus cells in media of different tonicities, but with [Na$^+$]$_i$ reduced to 25 mM. We used these conditions because others, using A6 cells, an epithelial cell line that exhibits both apical and basolateral NHE (31), have observed that pH$_i$ changes due to basolateral NHE are greatest at low Na$^+$ concentrations (32). Our findings of a substantial increase in pH$_i$ recovery in 0.95 × isotonic medium compared with isotonic low Na$^+$ medium are in accord with these data from A6 cells. The inhibition of MIA-sensitive pH$_i$ recovery in 0.70 × isotonic medium but with the same Na$^+$ concentration is consistent with reports of the effect of substantial hypotonicity on NHE in symmetrical cells. In nominally HCO$_3$-free medium, after acid loading, osteosarcoma cell suspensions underwent amiloride-sensitive pH$_i$ changes that are diminished in 0.70 × isotonic medium (9). Kinetic analysis of these data demonstrated that inhibition of the exchanger was due to decreased V$_{max}$ without a change in apparent affinity for H$^+$ or Na$^+$. Single cell analysis of ΔpH$_i$ after 0.70 × hypotonic dilution confirmed that NHE was inhibited following cell swelling (10). We have clearly shown that the MIA-sensitive pH$_i$ recovery from an acid load increased in 0.95 × isotonic medium, but decreased in 0.70 × isotonic medium compared with isotonic controls. We conclude that the activation of basolateral NHE-1...
during the modest 5–7% volume increase in the villus cells is not a consequence of the intracellular acidification normally observed after cell swelling.

Approximately 75% of the pH recovery from a moderate acid load in the villus cells was insensitive to the N-5-alkyl amiloride derivative. Our finding that bafilomycin inhibited 36% of the pH recovery suggests that a type V H⁺-ATPase contributes to pH homeostasis in these cells (33). This ATPase, which has been localized to the apical membrane of urinary bladder epithelial cells (34) and the plasma membrane of peritoneal macrophages (35), has been shown to contribute to pH recovery from acid loads in the presence of amiloride in both peritoneal (20) and alveolar (36) macrophages. We also observed that Zn²⁺, in the presence of this selective inhibitor of vacuolar H⁺-ATPase, further reduced by −39% the pH recovery of the villus cells. The concentration of Zn²⁺ used in our experiments has been shown by others to block H⁺ conductance in snail neurons (37) and human granulocytes (38). Furthermore, unequivocal results using transfected of NHE-1 have shown that H⁺ conductance, which is Zn²⁺-sensitive, may be dissociated from NHE activity and that substantial alkalinizations of pH due to NHE-1 still occur in the presence of ZnCl₂ (39). As such, the sensitivities of pH recovery from an acid load to bafilomycin and to Zn²⁺ suggest that both H⁺ conductance and a type V ATPase substantially contribute to pH homeostasis in villus cells.

Cellular acidification after cell swelling because of hypotonic dilution (0.70 to 0.50 × isotonic) has been observed in several symmetrical cell types. The source of acidification has been speculated to be conductive OH⁻ efflux through volume-activated Cl⁻ channels (10), increased glycolytic metabolic activity (8), or inhibited NHE activity (9). We found that cell swelling of 15% following this standard hypotonic dilution inhibited NHE activity. Clearly, several mechanisms contribute to swelling-induced cellular acidification since modest 5–7% volume increases similar to swelling caused by Na⁺ solute absorption cause villus cells to acidify as well as to activate NHE-1. Far from being a “housekeeping” function, the activation of NHE-1 may be an essential requirement for RVD following “physiological” volume increases, when these cells swell during Na⁺ solute absorption.

Alkalinizing the pH of cells swollen 5% in the presence of MIA by-passed inhibition and allowed complete RVD, whereas acidifying the pH had no effect on the inhibited volume reduction. The extent of this alkalinization, induced by NH₄Cl addition, was comparable to that observed in cells swollen after 0.95 × isotonic dilution. This finding suggests that Na⁺ influx resulting from activated NHE-1 is osmotically neutral and that it is the change in pH that is a determinant of the osmolyte loss (K⁺ and Cl⁻) required for volume regulation. Previously, we reported that RVD following swelling because of the uptake of D-glucose was sensitive to the high conductance Ca²⁺-activated

![FIG. 8. Effect of non-amiloride and amiloride analogues on RVD after 0.93 × isotonic dilution. A: ▲, clonidine (50 μM); ○, cimetidine (25 μM) (n = 7; p < 0.05). B: ●, clonidine; △, cimetidine; □, 5-(N,N-dimethyl) amiloride; ▼, MIA (n = 7). Volume was measured electronically and is expressed relative to the isotonic control.](http://www.jbc.org/content/214/52/23143/F1)

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(maxi-K) K⁺ channel blocker charybdotoxin, while RVD following a greater swelling of 15% caused by the standard 0.70 × isotonic dilution was insensitive to the toxin (3). Since the calcium gating of charybdotoxin-sensitive K⁺ conductance is exquisitely sensitive to alkaline pH_i (40), the activation of NHE-1 during the modest 5–7% volume increase may serve as the source of the required alkalinization for Ca²⁺ gating of the charybdotoxin-sensitive K⁺ loss. Because villus cells acidify as they swell 15% of their isotonic volume, we speculate that a different K⁺ conductance is activated for RVD following larger, “non-physiological” volume increases. As villus cells swell, the extent of that swelling is a key determinant of changes in pH_i, which in turn serve to signal the subsequent volume regulation.

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R. John MacLeod and J. Richard Hamilton

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