J. Membrane Biol. 172, 47–58 (1999)

The Journal of
Membrane
Biology

Increases in Intracellular pH and Ca^{2+} are Essential for K^+ Channel Activation After Modest 'Physiological' Swelling in Villus Epithelial Cells

R.J. MacLeod*, J.R. Hamilton

Department of Pediatrics, McGill University–Montreal Children's Hospital Research Institute, Montreal, Quebec, Canada

Received: 30 March 1999/Revised: 6 July 1999

Abstract. We studied the relationship between changes in intracellular pH (pH_i), intracellular Ca^{2+} ($[Ca^{2+}]_i$) and charybdotoxin sensitive (CTX) maxi-K^+ channels occurring after modest 'physiological' swelling in guinea pig jejunal villus enterocytes. Villus cell volume was assessed by electronic cell sizing, and pH_i and $[Ca^{2+}]_i$ by fluorescence spectroscopy with 2,7, biscarboxyethyl-5-6-carboxyfluorescein and Indo-1, respectively. In a slightly (0.93 × isotonic) hypotonic medium, villus cells swelled to the same size they would reach during D-glucose or L-alanine absorption; the subsequent Regulatory Volume Decrease (RVD) was prevented by CTX. After the large volume increase in a more hypotonic (0.80 × isotonic) medium, RVD was unaffected by CTX. After modest swelling associated with 0.93 × isotonic dilution, the pH_i alkalinized but N-5-methyl-isobutyl amiloride (MIA) prevented this ΔpH_i and the subsequent RVD. Even in the presence of MIA, alkalinization with added NH_4Cl permitted complete RVD which could be inhibited by CTX. The rate of ^{86}Rb efflux which also increased after this 0.93 × isotonic dilution was inhibited an equivalent amount by CTX, MIA or Na^+-free medium. Modest swelling transiently increased $[Ca^{2+}]_i$ and Ca^{2+}-free medium or blocking alkalinization by MIA or Na^+-free medium diminished this transient increase an equivalent amount. RVD after modest swelling was prevented in Ca^{2+}-free medium but alkalinization still occurred. After large volume increases, alkalinization of cells increased $[Ca^{2+}]_i$ and volume changes became sensitive to CTX. We conclude that both alkalinization of pH_i and increased $[Ca^{2+}]_i$ observed with 'physiological' volume increase are essential for the activation of CTX-sensitive maxi-K^+ channels required for RVD.

Key words: RVD — Calcium — pH — Charybdotoxin — K^+ channels — Villus epithelial cells

Introduction

In jejunal villus epithelial cells, it is the extent of cell swelling that determines signaling of the K^+ and Cl^- channels which in turn drive the Regulatory Volume Decrease (RVD) response to that swelling. High conductance Ca^{2+}-activated (maxi-K^+) K^+ channels which are sensitive to charybdotoxin (CTX) and essential for RVD after the small, 5–7% volume increases occurring during Na^+-solute absorption, but not for RVD after larger (15%) volume increases (MacLeod & Hamilton, 1991*a*,*b*; MacLeod, Lembessis & Hamilton, 1992). Having found a distinctive intracellular alkalinization associated with these modest 5 to 7% volume increases (MacLeod & Hamilton, 1996), we have studied the relationship between this alkalinization, altered intracellular calcium ($[Ca^{2+}]_i$) and K^+ channel activation after volume increases of 5 to 7%; we compared the response of cells subjected to this modest amount of swelling which replicates the response to Na^+-glucose absorption, with the response to the more substantial 12 to 15% volume increases routinely used in volume regulatory studies (Foskett, 1994; Lang et al., 1998).

Materials and Methods

Villus Epithelial Cell Isolation and Volume Determinations

Isolated from segments of adult male (200–300 g) guinea pig jejunum by mechanical vibration (MacLeod & Hamilton, 1990), villus cells

* *Present address:* Endocrine-Hypertension Division, Brigham and Women's Hospital, Department of Medicine, Harvard Medical School, 221 Longwood Avenue, Boston, MA 02115, USA

Correspondence to: R.J. MacLeod

were resuspended at 0.8–1.5 × 10^6 cells/mL in RPMI-1640 medium (without HCO_3) containing bovine serum albumin (type V) at 1 mg/mL and 20 mM Na Hepes, pH 7.3 at 37°C. Viability, assessed by trypan blue exclusion was 85% after 3 hr. Cell volume was measured using a Coulter Counter (Zm) and an attached Channelyzer (C-256) (MacLeod & Hamilton, 1991*a*,*b*). Villus cell volume measured electronically over a range of tonicities correlated positively ($r = 0.967$) with direct measurements of cell water (MacLeod & Hamilton, 1991*b*). Relative cell volume was expressed as the ratio of cell volume under experimental conditions to volume under basal conditions.

pH_i and $[Ca^{2+}]_i$ Measurements

pH_i was determined fluorometrically using BCECF. We loaded villus cell suspensions (1 × 10^6 cells/mL in Hepes RPMI) with BCECF by incubation with the parent acetoxymethyl ester (3.7 μM) for 15 min at 37°C. After washing, and resuspension, we placed 0.5 to 0.8 × 10^6 cells in 2 mL of medium and determined fluorescence using a Hitachi F-4000 spectrofluorometer with excitation at 495 nm and emission at 525 nm using 5 and 10 nm slits, respectively. Calibration was performed in K^+-rich medium using nigericin and a quench correction factor as described (Grinstein, Rothstein & Cohen, 1985). The resting pH_i of these cells in Hepes buffered RPMI-1640 medium (nominally HCO_3-free) was 7.39 ± 0.04, $n = 45$.

Intracellular calcium concentrations, $[Ca^{2+}]_i$, were measured fluorometrically using Indo-1. We loaded villus cell suspensions (1 × 10^6 cells/mL) in Hepes buffered RPMI supplemented with $MgCl_2$ (final concentration 1 mM) by incubation with the parent acetoxymethyl ester (3 μM) for exactly 15 min at 37°C. After washing and resuspension, 10^7 cells/mL were used for fluorescence determination in 1 mL of indicated buffer. The excitation and emission wavelengths were set at 331 nm and 410 nm using 3 nm and 10 nm slit widths, respectively. F_{max} was determined using Triton-X (final concentration, 0.1%) and F_{min} calculated using EGTA (2 mM) after determining autofluorescence. A dissociation contrast of 250 nM for the Indo-1-Ca^{2+} complex was used to calculate $[Ca^{2+}]_i$ as previously described (Powell et al., 1993).

^{85}Rb Efflux Measurements

We measured ^{86}Rb efflux from the villus cells using a technique we have previously described (MacLeod et al., 1992*c*). Villus cells (2 × 10^6 cells/mL) were loaded in RPMI medium with ^{86}Rb (10 μCi/mL) for 45 min. Cell suspensions were diluted fivefold with ^{86}Rb-free RPMI, centrifuged, and resuspended in Na^+ medium, pH 7.3, at 3–5 × 10^6 cells/mL. After one mL of this suspension was diluted isotonically 7% with Na^+ medium, 500 μL of the cell suspension were added to an equal volume of ice-cold isotonic ^{86}Rb-free Na^+-medium, which served as a 'stop' solution. An aliquot of this mixture was layered over 100 μL of a 3:2 (vol/vol) di-n-butyl-phthalate:di-*n*-nonylphthalate oil mixture and centrifuged at 1,300 × *g* for 20 sec using an Eppendorf microfuge to measure the amount of isotope in cells before hypotonic dilution. Then, after addition of the appropriate amount of distilled water to the remaining suspensions, aliquots of cell suspension were added to an ice-cold stop solution that was 0.93 × isotonic ^{86}Rb-free Na^+-medium. An aliquot was layered over oil and centrifuged as described above. Sampling was done in duplicate. Supernatant and oil were aspirated; the cell pellet was lyzed in 0.5 ml of 1% sodium dodecylsulfate. Radioactivity associated with the cell pellet was assessed with liquid scintillation counting. Results were expressed as a fraction of isotope remaining over time, compared with isotope in cells diluted isotonically.

Solutions

Cell volume measurements were made using 30,000 cells/mL in Na^+ medium which contained (in mM): 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 Hepes (pH 7.3, 295 mOsM/kg H_2O). K^+-rich medium contained NaCl, isosmotically replaced by KCl. Na^+-free medium was prepared by isosmotically replacing NaCl with the chloride salt of N-methyl-D-glucamine ($NMDG^+$). pH_i and $[Ca^{2+}]_i$ measurements were made in Na^+ medium, and where indicated, K^+-rich medium. Measured electronically, the volume of guinea pig villus cells suspended in isotonic medium (1,537 ± 86 fL; $n = 65$) was in accord with previous reports (MacLeod & Hamilton, 1990; MacLeod et al., 1992*c*).

Reagents

The acetoxymethyl ester of 2′, 7′ bis (carboxyethyl)5-(6)-carboxyfluorescein (BCECF) and Indo-1 were obtained from Molecular Probes (Eugene, OR). 5-N-methyl-N-isobutyl)-amiloride (MIA) and charybdotoxin (CTX) were from Research Biochemicals (Natick, MA) and $NMDG^+$ from Aldrich (Milwaukee, WI). Rp cAMPS was from Biolog Life Sciences (La Jolla, CA).

Statistics

Data were reported as means ± SE of five to fourteen experiments performed in duplicate. Differences in means were determined using Student's *t* test.

Results

Effect of Charybdotoxin (CTX) on RVD and Intracellular pH (pH_i) After Cell Swelling

We suspended villus epithelial cells in hypotonic medium, 0.93 × isotonic, to duplicate the 5 to 7% volume increase observed in response to D-glucose or L-alanine absorption, and determined the effect of the selective and potent inhibitor of maxi-K^+-channels, charybdotoxin (CTX) on subsequent RVD (Fig. 1). CTX (50 nM) prevented RVD in these cells (Fig. 1*A*); their final relative volume was larger than that of untreated controls (1.04 ± 0.01 *vs.* 1.00 ± 0.01, $P < 0.001$, $n = 5$). In contrast, villus cells which were hypotonically diluted 0.80 × isotonic, to cause a greater swelling, underwent RVD which was not affected by 50 nM CTX (Fig. 1*B*). Thus, after a 'physiological' volume increase of 5 to 7%, RVD must utilize a K^+ channel which is CTX-sensitive while greater volume increases utilize a K^+ channel which is not CTX-sensitive.

The resting pH_i of villus cells in nominally HCO_3-free medium was 7.39 ± 0.04, $n = 45$. After suspension in 0.93 × isotonic medium, the cells alkalinized (Fig. 2*A*): this alkalinization was blocked by 1 μM N-5-methyl-5-isobutyl-amiloride (MIA, ΔpH_i/3 min: 0.010 ± 0.010 pH units, $P < 0.002$, $n = 6$, Fig. 2*B*) but CTX (50 nM) had no effect on the alkalinization (ΔpH_i/3 min:

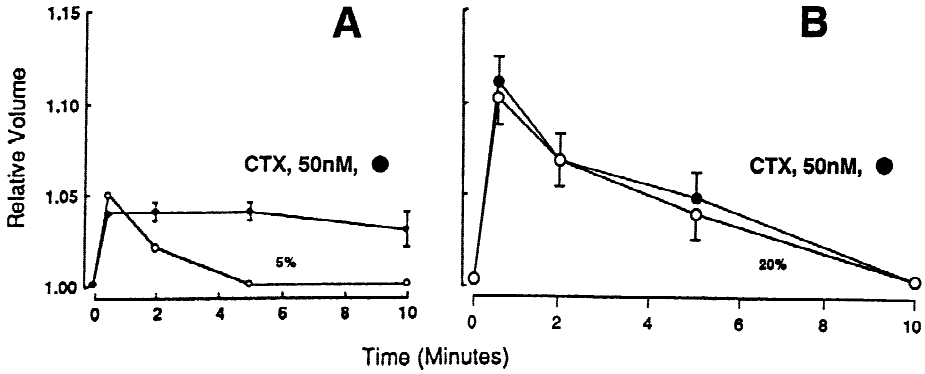

Fig. 1. Effect of charybdotoxin (CTX) on RVD after 0.95 × isotonic or 0.80 × isotonic dilution. (*A*) ●, 0.95 × isotonic, CTX (50 nM); ○, 0.95 × isotonic, $n = 4$. (*B*) ○, 0.80 × isotonic; ●, 0.80 × isotonic, CTX (50 nM), $n = 4$. Volume measured electronically, expressed relative to isotonic control.

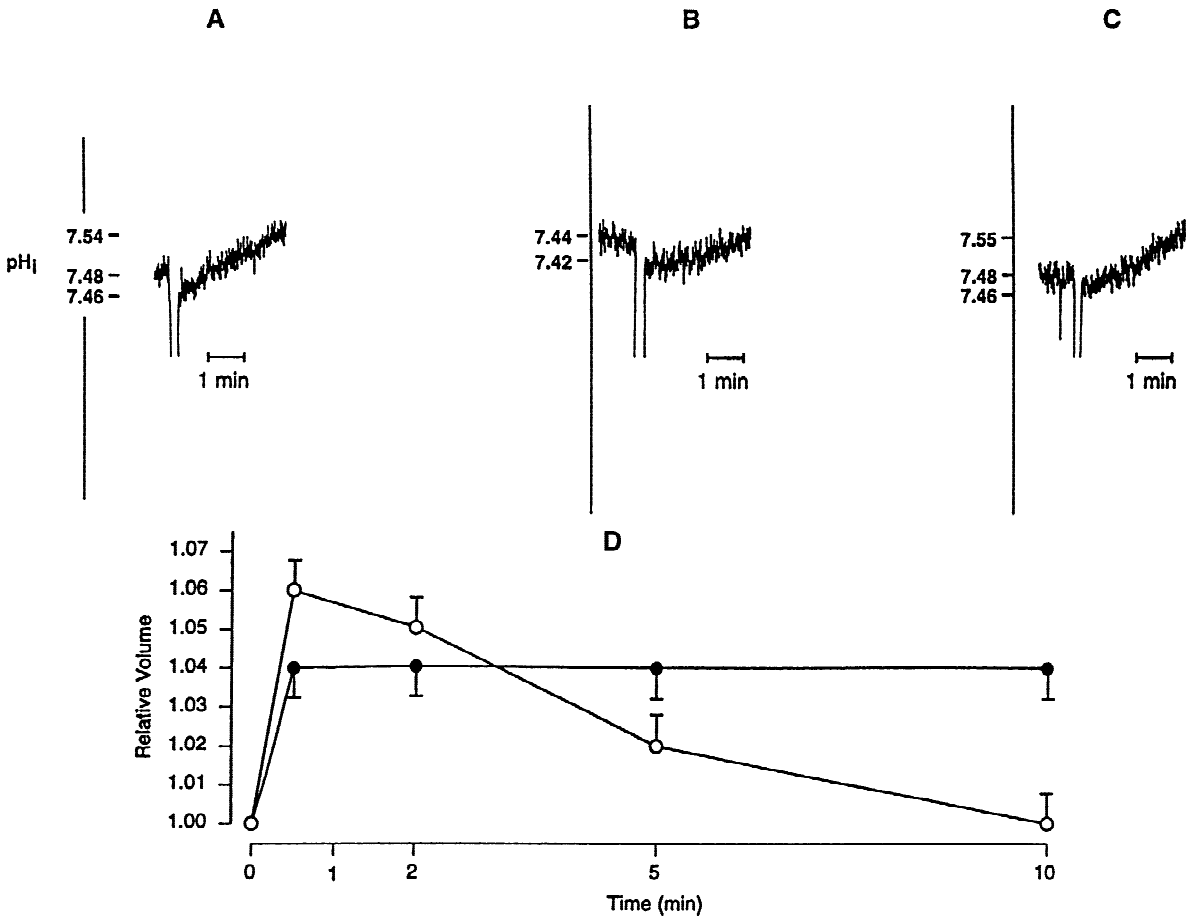

Fig. 2. Changes in pH_i and volume after 0.93 × isotonic dilution. (*A*) Alkalinization after 7% hypotonic dilution. (*B*) Effect of 1 μM N-5-methyl-isobutyl amiloride (MIA). (*C*) Effect of CTX (50 nM) on ΔpH_i after 7% hypotonic dilution. (*D*) Effect of MIA (1 μM) on RVD after 7% hypotonic dilution. 0, 0.93 × isotonic; ●, MIA (1 μM) + 0.93 × isotonic. $N = 6$ for all experiments. pH_i tracings of a single experiment are illustrated.

0.080 ± 0.10 *vs.* 0.070 ± 0.010 pH units, $n = 6$; Fig. 2*C*). Subsequent RVD was also prevented by the amiloride derivative (Fig. 2*D*); the final relative volume of cells was larger in the presence of 1 μM MIA compared with untreated controls (1.04 ± .01 *vs.* 1.00 ± .01, $n = 6$, $P < 0.001$). Amiloride-sensitive alkalinization seen after modest volume increase seems to be required to activate the K^+ or Cl^- channels necessary for RVD.

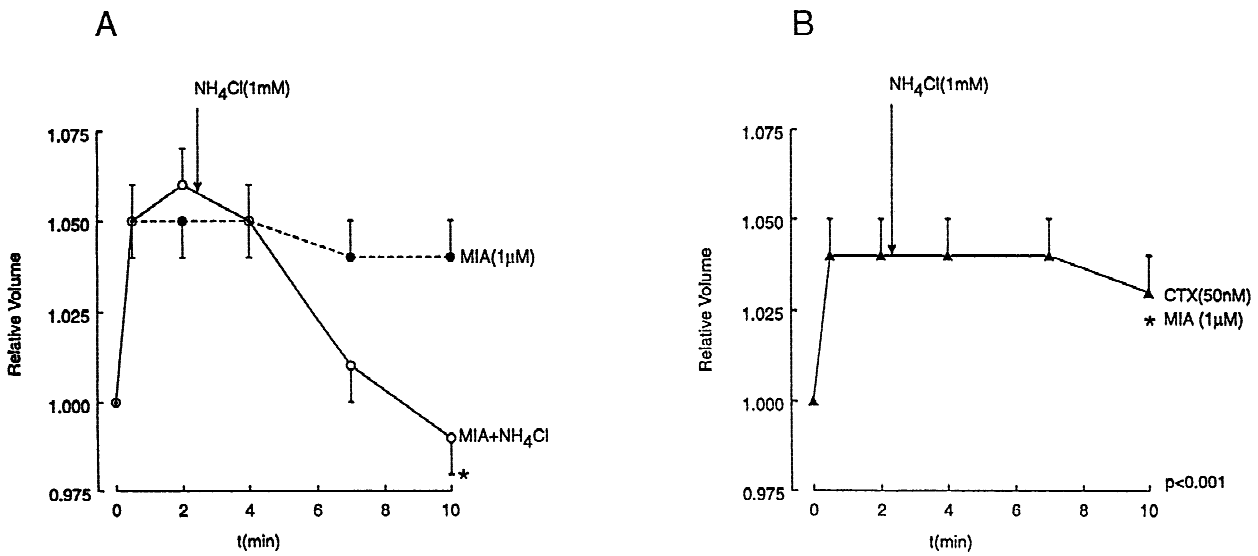

Fig. 3. Effect of CTX on NH_4Cl bypass of methyl-isobutyl-amiloride inhibition of RVD. (*A*) ●, 0.93 × isotonic, 1 μM MIA; ○, 0.95 × isotonic, 1 μM MIA, 1 mM NH_4Cl, $n = 6$. (*B*) ▲, CTX (50 nM), MIA (1 μM), NH_4Cl (1 mM), 0.93 × isotonic, $n = 6$. (*C*) Representative pH_i tracing a single experiment showing addition of 1 mM NH_4Cl to cells after 0.93 × isotonic dilution in the presence of 1 μM MIA. *$P < 0.001$.

This prevention of RVD by 1 μM MIA was bypassed by 1 mM NH_4Cl which caused an alkalinization of 0.080 ± 0.010 pH units ($n = 6$) and resulted in complete volume recovery (Fig. 3*A*). The final relative volume of cells treated with NH_4Cl and MIA was less than cells treated with MIA alone (0.99 ± .01 *vs.* 1.04 ± .01, $n = 6$, $P < 0.001$). In contrast, the addition of CTX (50 nM) to cells hypotonically diluted 7% in the presence of MIA and 1 mM NH_4Cl, prevented RVD (Fig. 3*B*). The final relative volume of CTX treated cells was larger than those treated with MIA and NH_4Cl without CTX (1.03 ± .01 *vs.* 0.99 ± .01, $n = 6$, $P < 0.001$). These findings suggested to us that the amiloride-sensitive alkalinization after 7% cell swelling must precede the activation of the CTX-sensitive K^+ channel required for RVD.

Effect of CTX, N-5-methyl-5-isobutyl-amiloride or Na^+-free Medium on ^{86}Rb Efflux

When cells were suspended in a 0.93 × isotonic medium the rate of ^{86}Rb loss increased compared with the loss from control cells in an isotonic medium (fraction of ^{86}Rb lost/5 min: 0.29 ± 0.03 *vs.* 0.17 ± 0.03, $P < 0.025$) (Fig. 4). CTX completely inhibited this increased rate (fraction of ^{86}Rb lost/5 min: 0.18 ± 0.02, $P < 0.02$, $n = 5$), as did MIA (fraction of ^{86}Rb lost/5 min: 0.17 ± 0.04, $P < 0.02$, $n = 5$) and a Na^+-free medium (fraction of ^{86}Rb lost/5 min: 0.19 ± 0.01, $P < 0.02$, $n = 5$). There were no differences in rates of ^{86}Rb loss when the effect of CTX was compared with MIA or Na^+-free conditions. Based on these results, we suggest that it is the CTX-sensitive K^+ channel that responds to MIA-sensitive alkalinization, since if it were the Cl^- channel, MIA or Na^+ free medium would have little effect on ^{86}Rb loss after the 0.93 × isotonic dilution.

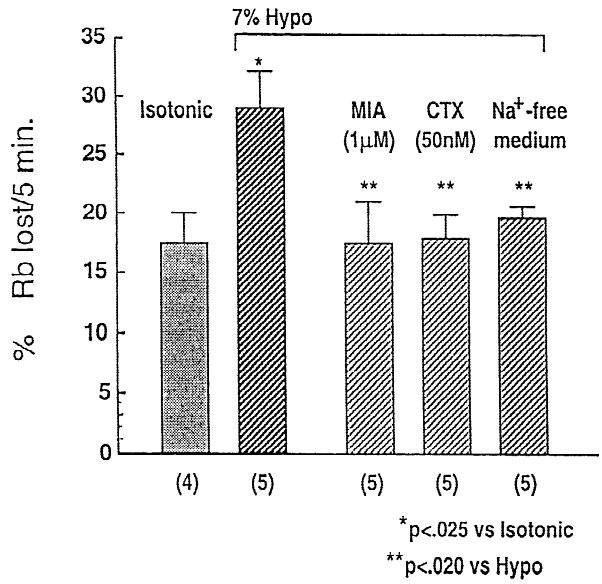

Fig. 4. Effect of CTX, N-methyl-isobutyl-amiloride (MIA), Na^+-free medium on ^{86}Rb efflux after 7% hypotonic dilution. MIA (1 μM); CTX (50 nM); Na^+-free medium prepared by isotonic replacement of NaCl with N-methyl-D-glucamine Cl. **$P < 0.02$ *vs.* 7% hypotonic, *$P < 0.025$ *vs.* isotonic. Results of 4 to 5 experiments performed in duplicate.

Effect of 7% Hypotonic Dilution, MIA and Na^+-Free Medium on $[Ca^{2+}]_i$

We determined the response of $[Ca^{2+}]_i$ to cell swelling after a 7% hypotonic dilution as well as in the presence

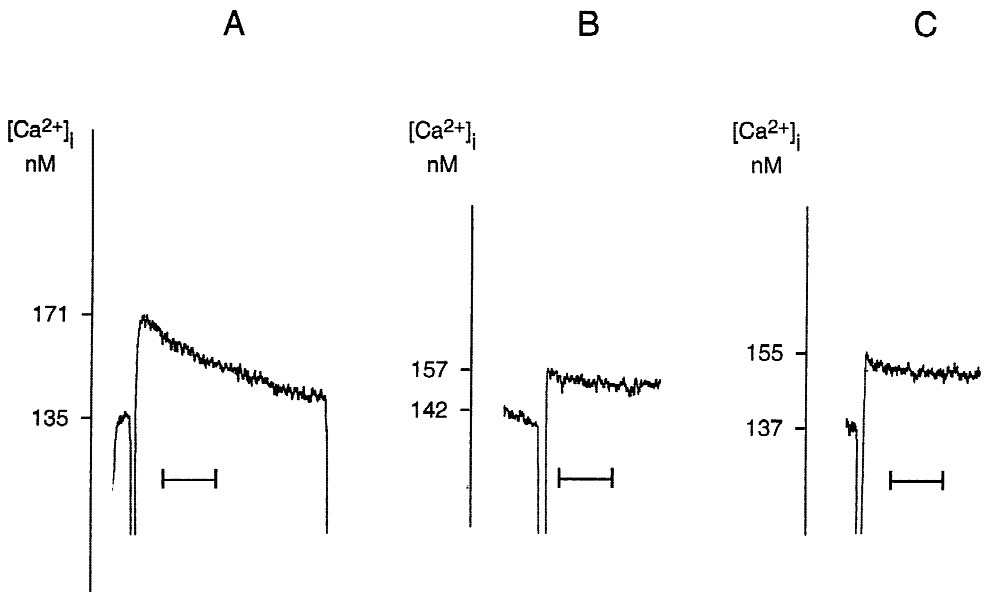

Fig. 5. Effect of 0.93 × isotonic dilution, MIA or Na^+-free medium on $[Ca^{2+}]_i$. (*A*) Control, 0.93 × isotonic dilution at break in trace. Bar represents 60 sec. (*B*) Effect of 0.93 × isotonic dilution in medium containing 1 μM MIA. Bar represents 60 sec. (*C*) Effect of 0.93 × isotonic dilution in Na^+-free medium. Traces from one experiment. For *n* of each treatment *see* Results.

of MIA (1 μM) or Na^+-free medium ($NMDG^+$), two treatments that attenuate the rate of ^{86}Rb efflux from these cells after modest swelling (Fig. 5). The resting level of $[Ca^{2+}]_i$ in villus cells suspended in control isotonic Na^+-containing medium was 144 ± 9 nM, $n = 14$. A 7% hypotonic dilution caused a rapid increase of 33 ± 6 nM ($P < 0.05$) which by 3 min declined to 12 ± 5 nM, $P < 0.05$ above the resting $[Ca^{2+}]_i$ (Fig. 5*A*). In the presence of MIA this rapid $[Ca^{2+}]_i$ increase was reduced to 18 ± 7 nM ($P < 0.001$ *vs.* control), but it was sustained at 15 ± 6 nM, $n = 14$, above resting $[Ca^{2+}]_i$ (Fig. 5*B*). In Na^+-free medium, the initial $[Ca^{2+}]_i$ increase was also less than control, 16 ± 6 nM, ($P < 0.001$, $n = 14$), and sustained at 15 ± 7 nM above resting levels (Fig. 5*C*).

Effect of Extracellular Ca^{2+} Depletion on Volume Regulation, $[Ca^{2+}]_i$ and pH_i

We then measured the volume, $[Ca^{2+}]_i$ and pH_i response of villus cells to a 7% hypotonic dilution in nominally Ca^{2+}-free (150 μM EGTA) medium (Fig. 6). The basal level of $[Ca^{2+}]_i$ was reduced (74 ± 8 nM, $P < 0.001$, $n = 12$) and the RVD normally seen in response to 7% hypotonic dilution was prevented (Fig. 6*A*). The final relative volume of these cells was larger than that of cells in Ca^{2+} (1 mM) containing medium (1.04 ± .01 *vs.* 1.00 ± 0.01, $P < 0.001$, $n = 6$). The initial $[Ca^{2+}]_i$ increase observed with hypotonic dilution was substantially reduced compared with controls (18 ± 1 *vs.* 33 ± 6 nM, $P < 0.001$, $n = 7$) and $[Ca^{2+}]_i$ continued to decline (Fig. 6*B*); 7% hypotonic dilution in this medium caused an alkalinization (ΔpH_i/3 min: 0.080 ± 0.010 pH units; Fig. 6*C*) which was blocked by 1 μM MIA (ΔpH_i/3 min: 0.010 ± 0.010 pH units, $P < 0.002$, $n = 8$). Thus, while modest volume increase in Ca^{2+}-free medium still allows activation of amiloride-sensitive NHE, the rise in $[Ca^{2+}]_i$ was diminished and RVD prevented. Activation of CTX-sensitive K^+ channels requires both an increase in $[Ca^{2+}]_i$ of ≥27 nM and pH_i of ≥0.07 pH units for complete RVD after a modest volume increase.

Alkalinizing pH_i Activates CTX-sensitive Secondary Cell Swelling

To determine whether activation of CTX-sensitive K^+ channels required an alkaline pH_i, we first generated volume increases where CTX-insensitive K^+ channels were activated (volume increases >15% after 0.5 × isotonic dilution), then made the pH_i more alkaline and assessed whether subsequent volume changes were CTX-sensitive. The rationale for this approach was that the direction and extent of volume regulation of these cells had been shown to be driven by the extracellular K^+ gradient, so that in 70 mM K^+ (final) these cells remained at the size they reached after hypotonic dilution, but at higher K^+, the cells continued to swell or exhibit 'secondary' swelling (MacLeod & Hamilton, 1991*b*). After suspending cells in 0.5 × isotonic K^+-rich medium, the addition of 50 mM NH_4Cl (Fig. 7*A*) resulted in an alkalinization of ≃0.39 pH units. This maneuver resulted in a sustained secondary cell swelling (Fig. 7*b*) (rel. vol.: 1.25 ± .01 *vs.* 1.16 ± .01, $n = 5$, $P < 0.05$), which was

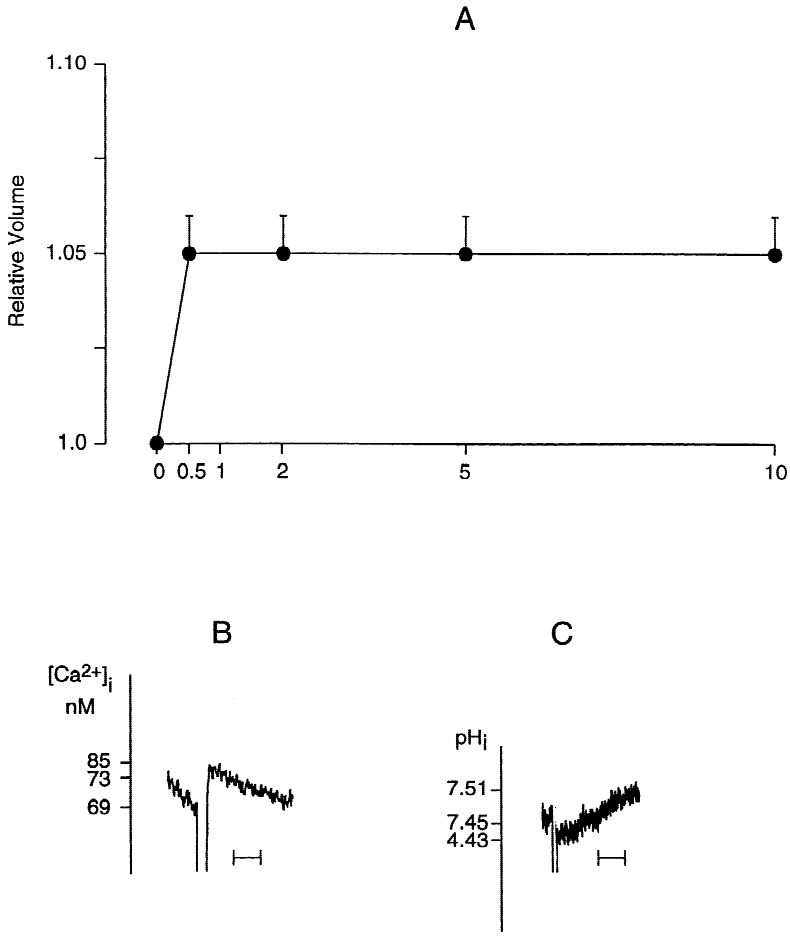

Fig. 6. Effect of Ca^{2+}-free (150 μM EGTA) medium on RVD, $[Ca^{2+}]_i$ and pH_i after 0.93 × isotonic dilution. (*A*) ●, 0.93 × isotonic, $n = 5$. Volume measured electronically, expressed relative to control. (*B*) $[Ca^{2+}]_i$ changes after 0.93 × isotonic dilution at break in trace. Bar represents 60 sec, $n = 8$. (*C*) pH_i changes after 0.93 × isotonic dilution. Bar represents 60 sec. Traces from one experiment. $n = 8$.

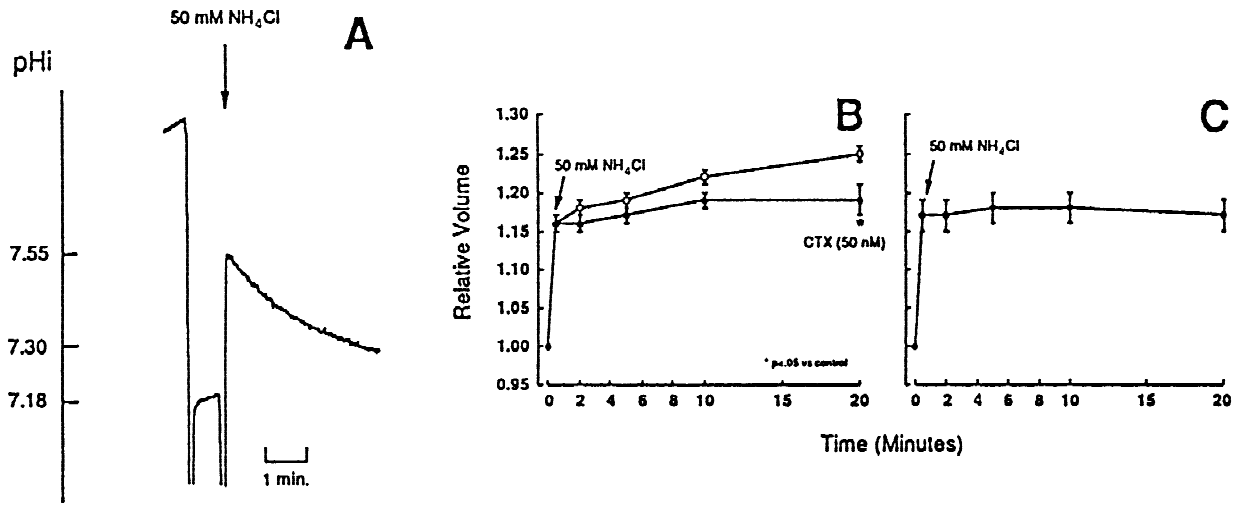

Fig. 7. Alkalinization of pH_i caused CTX-sensitive secondary volume changes: (*A*) ΔpH_i after addition of 50 mM NH_4Cl to cells suspended in K^+-rich medium, diluted 0.5 × isotonic. Tracing not corrected for dilutional artifact. (*B*) Cell volume changes in response to these manipulations. ●, CTX (50 nM), 0.5 × isotonic, 50 mM NH_4Cl. ○, 0.5 × isotonic, 50 mM NH_4Cl. (*C*) ●, 0.5 × isotonic, 50 mM NH_4Cl in Ca^{2+} free (150 μM EGTA) K^+-rich medium. $n = 5$. $^*P < 0.05$ *vs.* control.

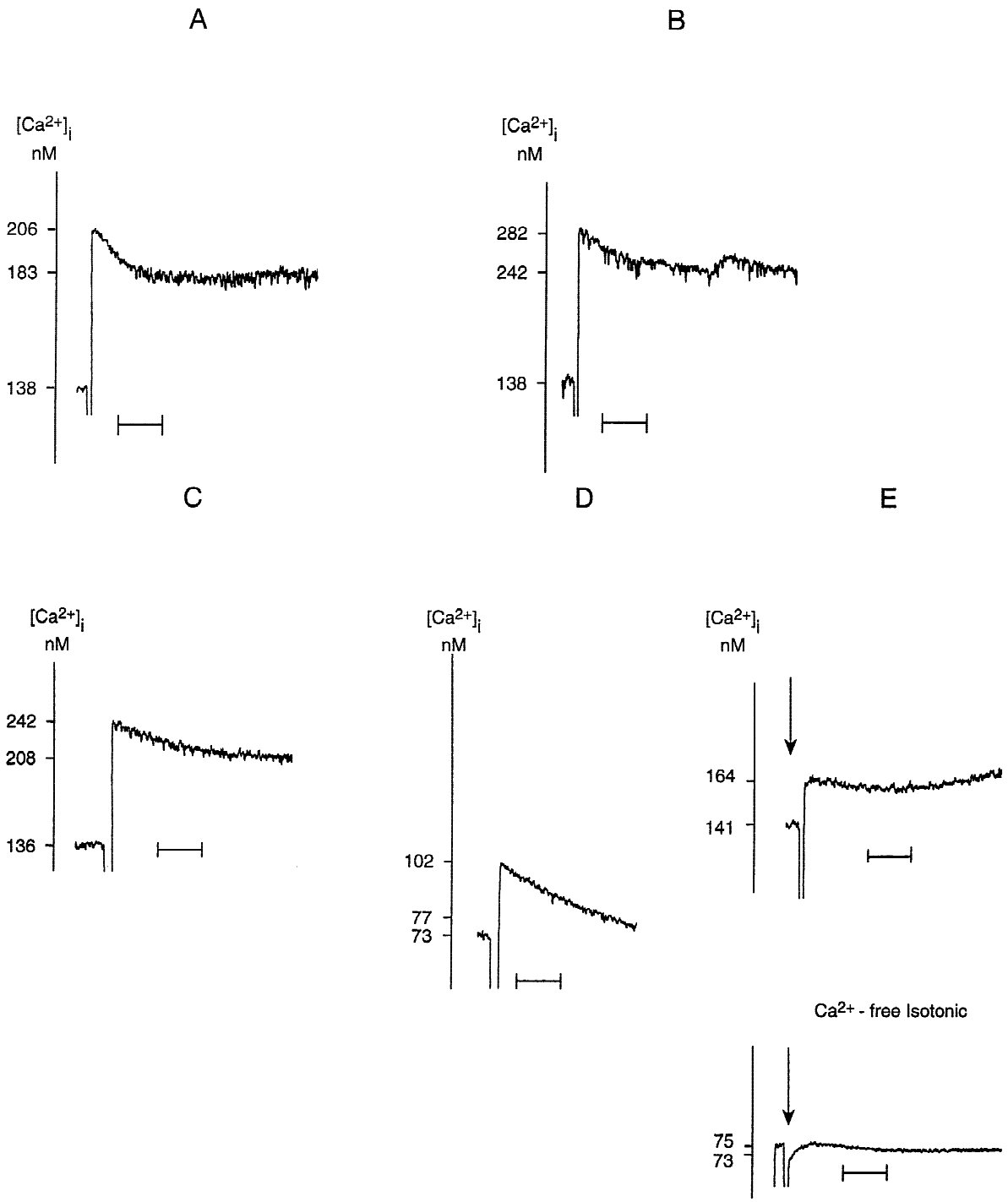

Fig. 8. Effect of NH_4Cl addition on $[Ca^{2+}]_i$ during substantial (50%) hypotonic dilution. (*A*) Villus cells suspended in K^+-rich medium. Break in trace is opening of compartment and hypotonic dilution. $[Ca^{2+}]_i$ rapidly increases then falls to a sustained plateau. Bar indicated 60 sec. (*B* and *C*) NH_4Cl (50 mM, final) added during hypotonic dilution. Decline of initial increase of $[Ca^{2+}]_i$ slower and elevated plateau higher than untreated cells (*see* Results). (*D*) NH_4Cl addition in nominally Ca^{2+}-free (150 μM EGTA) K^+-rich medium. (*E*) Upper trace-NH_4Cl (50 mM) addition to cells in isotonic K^+-rich medium. Lower trace-NH_4Cl addition to cells in isotonic nominally Ca^{2+}-free (150 μM EGTA) K^+-rich medium. Traces representative of one experiment. For *n* of each treatment *see* Results.

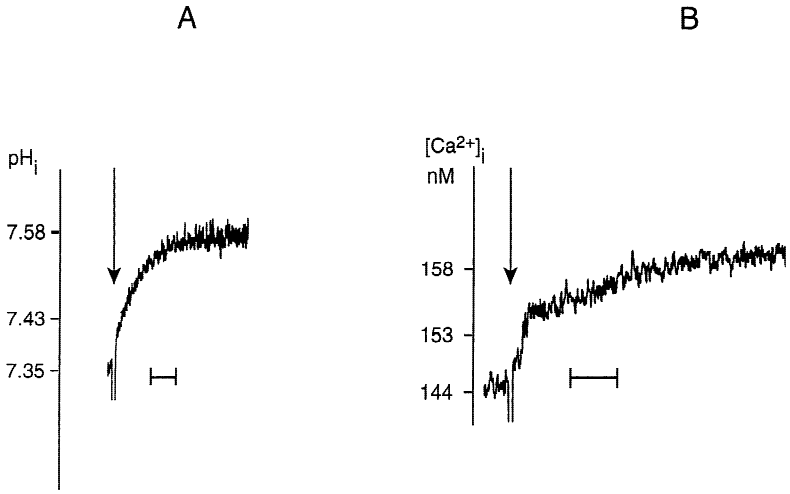

Fig. 9. Effect of monensin (10 μM), a Na^+/H^+ exchanger on pH_i and $[Ca^{2+}]_i$. (*A*) pH_i changes after monensin addition in isotonic Na^+ (140 mM) medium (arrow). Bar indicates 60 sec. (*B*) $[Ca^{2+}]_i$ changes after monensin addition in isotonic Na^+ (140 mM) (arrow). Bar indicates 60 sec. Traces representative of one experiment. $n = 8$.

completely prevented by CTX (50 nM) (final rel. vol. 1.19 ± 0.02, $n = 5$, $P < 0.05$). In nominally Ca^{2+}-free medium, containing 150 μM EGTA, there was no secondary swelling response to alkalinization (Fig. 7*C*) (rel. vol. 1.17 ± .02 *vs.* 1.17 ± .02) suggesting that an increase in $[Ca^{2+}]_i$ from an extracellular source was required, in addition to the alkalinization, to activate CTX-sensitive secondary swelling.

Effect of Intracellular Alkalinization on $[Ca^{2+}]_i$

The addition of NH_4Cl to cells swollen in K^+-rich medium caused a transient alkalinization but a persistent secondary cell swelling (Fig. 7). To assess whether this persistent swelling in spite of a transient ΔpH_i was due to a sustained increase in $[Ca^{2+}]_i$, we measured $[Ca^{2+}]_i$ in response to a substantial (0.5 × isotonic) challenge in K^+-rich medium, in response to 50 mM NH_4Cl added simultaneously with the hypotonic challenge, and then in comparable conditions but in Ca^{2+}-free K^+-rich medium (Fig. 8). Basal $[Ca^{2+}]_i$ of cells in isotonic K^+-rich medium containing 1 mM Ca^{2+} (final) was 139 ± 9 nM, $n = 32$. A 0.5 × isotonic dilution caused a rapid increase to 220 ± 12 nM, $n = 14$, which over the next 60 ± 8 sec relaxed to a plateau that averaged 175 ± 9 nM (Fig. 8*A*). This elevated plateau was maintained for 5 min. Addition of 50 mM NH_4Cl with the 0.5 × isotonic dilution caused a higher initial increase which took longer to relax to a sustained plateau (Fig. 8*b* and *c*). The initial increase, 143 ± 21 nM *vs.* 81 ± 16 nM, ($P < 0.05$, $n = 14$), was higher than for controls not treated with NH_4Cl. The decline of this peak to the sustained plateau took 120 ± 32 sec, ($P < .05$), longer than untreated controls and $[Ca^{2+}]_i$ was maintained 133 ± 17 nM, $P < 0.05$, above basal levels which was higher than cells not treated with NH_4Cl. Suspended in nominally Ca^{2+}-free (150 μM EGTA) K^+-rich medium, the villus cells had a basal level of $[Ca^{2+}]_i$ (73 ± 8 nM, $P < 0.001$, $n = 12$) which was lower than in Ca^{2+}-containing medium. Addition of NH_4Cl (50 mM) during the 0.5 × isotonic dilution of these cells caused an initial transient rise of 17 ± 9 nM, ($P < 0.001$, $n = 12$), which was substantially lower than in Ca^{2+}-containing medium (Fig. 8*D*). There was no sustained plateau; within ~3 min $[Ca^{2+}]_i$ had returned to basal levels. Clearly, extracellular Ca^{2+} was required for the NH_4Cl induced increase in $[Ca^{2+}]_i$. In isotonic K^+-rich medium addition of NH_4Cl (50 mM) rapidly increased $[Ca^{2+}]_i$ 21 ± 4 nM, whereas in Ca^{2+}-free (150 μM EGTA) isotonic K^+-rich medium this increase was prevented (1 ± 1 nM, $n = 6$, $P < 0.05$, Fig. 8*E*).

Because this rapid increase in $[Ca^{2+}]_i$ caused by NH_4Cl addition required extracellular Ca^{2+}, we used an alternative mechanism to increase the pH_i of villus cells suspended in isotonic Na^+ (140 mM) medium to assess its effect on villus cell $[Ca^{2+}]_i$. Monensin (10 μM), a Na^+/H^+ exchanger, when added to the cells in suspension caused a rapid alkalinization of the cells which reached a steady state ~3.5 min after addition (Fig. 9*A*). Within 30 sec of addition, the pH_i was 0.05 ± 0.01 pH units ($P < 0.05$) higher and at three minutes, it was 0.23 ± 0.03 pH units higher ($P < 0.001$, $n = 8$) than resting pH_i. Intracellular $[Ca^{2+}]_i$ (Fig. 9*b*) at 30 sec was 7 ± 2 nM ($P < 0.05$, $n = 8$), greater and at 3 min it was 12 ± 1 nM ($P < 0.05$, $n = 8$), higher than basal levels. In nominally Ca^{2+}-free (150 μM EGTA) medium, the increase in $[Ca^{2+}]_i$ within 3 min of monensin addition was blocked (0 ± 1 nM, $P < 0.05$, $n = 8$). This finding added further support to the concept that alkalinization of pH_i increases $[Ca^{2+}]_i$ by rapidly mobilizing extracellular Ca^{2+}.

Effect of PKA Inhibition on 0.93 × Isotonic RVD

Since some CTX-sensitive maxi K^+ channels may be activated by phosphorylation via protein kinase A (Rein-

hart et al., 1991) we determined whether an inhibitor of PKA might alter CTX-sensitive RVD. The addition of 8-Br-cAMP (0.5 mM) to the villus cells in isotonic medium resulted in a slight volume reduction (final relative vol. 0.95 ± .01, $P < 0.05$, $n = 6$). In the presence of the PKA antagonist, Rp-cAMPS (10 μM), the 8-Br-cAMP stimulated volume reduction was prevented (final relative vol. 1.00 ± .01 *vs.* 0.95 ± .01, $P < 0.05$, $n = 6$). This PKA antagonist had no effect on RVD after a 7% hypotonic dilution. The extent of the volume decrease (6 ± 1%) was no different in the presence of Rp-cAMPS (6 ± 1%).

Discussion

The current studies further characterize the distinctive volume regulatory mechanisms activated by modest 5–7% cell swelling occurring after exposure to a slightly hypotonic (0.93–0.95 × isotonic) medium. This model of modest swelling was created to duplicate the extent of swelling when these cells absorb D-glucose or L-alanine (MacLeod & Hamilton, 1991; MacLeod et al., 1992*a*,*b*). The charybdotoxin (CTX) sensitive K^+ channel activated by this modest presumably 'physiological' volume increase requires intracellular alkalinization as shown by the inhibitory responses to a N-5-alkyl amiloride derivative and to Na^+-free medium, and by the volume responses to NH_4Cl-induced alkalinization after such inhibition. This alkalinization, which we have previously characterized as due to the hypotonic activation of the NHE-1 isoform of Na^+/H^+ exchange (MacLeod & Hamilton, 1996), increases the initial transient $[Ca^{2+}]_i$ increase which occurs with a modest swelling. The extent of the initial $[Ca^{2+}]_i$ increase was diminished by an amiloride derivative or by Na^+-free medium, and both maneuvers prevent Regulatory Volume Decrease (RVD). Furthermore, depletion of extracellular Ca^{2+} diminished the initial $[Ca^{2+}]_i$ increase after a modest swelling and blocked RVD but had no effect on the alkalinization. We conclude that both an increase in pH_i and in $[Ca^{2+}]_i$ are required after modest 'physiological' swelling of villus epithelial cells for the activation of the CTX-sensitive K^+ channels utilized for this pathway of volume regulation.

High conductance Ca^{2+}-activated K^+ (maxi-K^+) channels have single-channel conductances >100 pS (Latorre et al., 1989) which CTX, a peptidyl toxin, blocks with high affinity in several epithelia (Cornejo, Guggino & Guggino, 1984; Tanc et al., 1993; Lu, Markakis & Guggino, 1993; Garcia et al., 1995). Other maxi-K^+ channels are insensitive to the toxin (Reinhart et al., 1991). Single-channel analyses of maxi-K^+ channels (MacKinnon & Miller, 1988; Klaerke et al., 1993) have suggested that CTX physically plugs the channels' externally facing pore and several reports have suggested maxi-K^+ channel activation during RVD (Christensen, 1987; Hazama & Okada, 1990; Suzuki et al., 1990; Foskett, 1994; Park et al., 1994). There are three mechanisms by which maxi-K^+ channel activity can be modulated: pH_i, Ca^{2+} (Christensen & Zeuthen, 1987) and protein phosphorylation (Reinhart et al., 1991). Acidification at a fixed Ca^{2+} concentration can decrease maxi-K^+ channel activity (Cook, Ikeuchi & Fujimoto, 1984; Christensen & Zeuthen, 1987; Cornejo et al., 1989; Copello, Segal & Reuss, 1991; Laurido et al., 1991; Klaerke et al., 1993). Alkalinization in a physiological range will increase maxi-K^+ channel activity (Copello et al., 1991; Andersen et al., 1995). Our data indicate that alkalinization caused by the activated NHE-1 (MacLeod & Hamilton, 1996) precedes activation of the CTX-sensitive K^+ channel after 5–7% swelling in enterocytes. The increased rates of ^{86}Rb efflux associated with RVD were inhibited by either the amiloride derivative or by Na^+-free medium, both conditions which blocked intracellular alkalinization. RVD did proceed when sufficient NH_4Cl was added to override this inhibition but it was inhibited still by CTX. We conclude that alkalinization is required for the activation of CTX-sensitive K^+ channels utilized for RVD in jejunal villus enterocytes after a modest 5 to 7% volume increase.

The current studies support the conclusion that alkalinization increases $[Ca^{2+}]_i$ of villus epithelial cells. The addition of NH_4Cl to cells in isotonic or hypotonic medium significantly raised $[Ca^{2+}]_i$; activating alkalinization by adding the Na^+/H^+ exchanger, monensin, also caused a rapid increase in $[Ca^{2+}]_i$, and blocking alkalinization after a modest volume increase reduced the extent of the initial $[Ca^{2+}]_i$ increase. Irrespective of the source of alkalinization, the increases in $[Ca^{2+}]_i$ were attenuated in nominally Ca^{2+}-free media suggesting to us that alkalinization activates a Ca^{2+} influx pathway in villus cells. In other nonepithelial cell types, intracellular alkalinization has been shown to increase Ca^{2+} current or open channel probability of 'L' type Ca^{2+} channels. Acidification has the opposite effect (Klöckner & Isenberg, 1994; Yamakage et al., 1995; Shmigol et al., 1995). Indeed, angiotensin II-induced increase of L-type Ca^{2+} channels required alkalinization and was attenuated by amiloride or removal of extracellular Na^+ (Kaibara et al., 1994). In the current experiments, volume increases of ≥12% after 0.5 × isotonic dilution were insensitive to CTX, yet directly stimulating a transient alkalinization of villus cells resulted in continued cell swelling which was CTX-sensitive and dependent on the presence of extracellular Ca^{2+}. Maxi-K^+ channels require an increase in $[Ca^{2+}]_i$ for activation (Latorre et al., 1989) so the observed, continued swelling of cells after transient alkalinization was consistent with the view that increased $[Ca^{2+}]_i$ of the alkalinized cells had activated CTX-sensitive K^+ channels. The absence of CTX-sensitive

swelling in Ca^{2+}-free medium of alkalinized cells was consistent with a lower $[Ca^{2+}]_i$ level being insufficient for the activation of these channels.

In the current experiments, hypotonic activation of NHE-1 caused sufficient alkalinization to increase $[Ca^{2+}]_i$ and to activate CTX-sensitive K^+ channels leading to complete RVD. Both modest (7%) and substantial (15–20%) volume increases caused a pattern of changes in $[Ca^{2+}]_i$ similar to those reported in other epithelia (Hazama & Okada, 1990; McCarty & O'Neil, 1991, 1992; Foskett, 1994). Hypotonic dilution generates an increase in $[Ca^{2+}]_i$ which is proportionate to the amount of hypotonicity. The initial transient increase of $[Ca^{2+}]_i$ may be due to Ca^{2+} influx (Ross & Cahalen, 1995) or Ca^{2+} release from nonmitochondrial stores (Missiaen et al., 1996). We observed that blocking the hypotonic activation of NHE-1 by MIA or Na^+-free medium reduced the initial $[Ca^{2+}]_i$ increase an equivalent amount. In nominally Ca^{2+}-free medium, this initial $[Ca^{2+}]_i$ increase was also diminished but the hypotonic activation of NHE-1 still occurred. The comparability of inhibiting NHE-1 and Ca^{2+}-free conditions on the extent of initial $[Ca^{2+}]_i$ increase suggested to us that alkalinization from NHE-1 was mobilizing Ca^{2+} influx from an extracellular source. We conclude that the activation of CTX-sensitive K^+ channels for RVD requires both an increase of $[Ca^{2+}]_i$ of $\geq$27 nM and an alkalinization of pH_i of $\geq$0.07 pH units above basal levels. This increase in pH_i from the hypotonically activated NHE-1 serves to increase $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from an extracellular source.

Our current experiments suggest that for activation of the CTX-sensitive K^+ channel for RVD after modest swelling there is a critical $[Ca^{2+}]_i$ threshold as well as an absolute requirement for NHE-1 activation. This $[Ca^{2+}]_i$ requirement is exquisitely sensitive, since an increase to ~157 nM (in the presence of MIA or Na^+-free medium) did not activate CTX-sensitive ^{86}Rb efflux while ~177 nM did. This level is in agreement with the 200 nM $[Ca^{2+}]_i$ threshold required to activate CTX-sensitive K^+ channels in human leukemic T cells and resting lymphocytes (Grissmer, Lewis & Cahalan, 1992; Grissmer, Nguyen & Cahalan, 1993). Because acidification will inhibit the CTX-sensitive K^+ channel and volume increases >7% result in an intracellular acidification (MacLeod & Hamilton, 1996), the CTX-insensitivity of RVD after substantial volume increases may be due to this acidification. The role of $[Ca^{2+}]_i$ changes and intracellular acidification in activating K^+ channels after volume increases of >7% are addressed in a separate study (MacLeod & Hamilton, 1999).

Our current experiments which support an essential role for Ca^{2+} in RVD of villus epithelial cells are in contrast with studies using other cell types (Altamirano, Brodwick & Alvarez-Leefmans, 1998). Loading villus cells with the intracellular calcium chelator BAPTA prevented RVD after a modest 5% volume increase (MacLeod et al., 1992*a*. In the current studies, we report that when extracellular calcium is depleted using EGTA and Ca^{2+}-free medium or the activation of NHE-1 is blocked, the initial rise in $[Ca^{2+}]_i$ that accompanies this modest volume increase is diminished an equivalent amount. Both maneuvers prevent RVD but in Ca^{2+}-free medium, alkalinization still occurs. Therefore, we conclude that the Ca^{2+} increase is necessary for RVD and not an epiphenomenon. Indeed, our current studies not only suggest a causal relationship for calcium increases and RVD in this model of cell swelling but define a critical threshold of $[Ca^{2+}]_i$ and pH_i increases required for CTX-sensitive K^+ channel activation leading to successful RVD.

The cell permeant inhibitor of cAMP-dependent protein kinase A, Rp-cAMPS (Rothermel, Botelho & Parker, 1988) had no effect on RVD after the modest swelling observed with 0.93 × isotonic dilution. This finding suggested to us that the CTX-sensitive K^+ channel required for this RVD is not activated by protein kinase A. Previously we have reported that different inhibitors of protein kinase C have no effect on RVD under similar conditions (MacLeod et al., 1992*b*). Together these results suggest neither PKA nor PKC activate the CTX-sensitive K^+ channel responsible for RVD after modest volume increases in the villus cells.

Our results indicate that the relationship between the extent of villus cell swelling and class of K^+ channel activated for subsequent RVD is attributable to very different pH_i responses to volume increases of differing magnitude. Studies using mammalian lymphocytes first speculated that the pH sensitivity of the K^+ conductance activated for RVD made pH_i a putative regulator of RVD, without identifying whether pH_i changed with volume increases (Deutsch & Lee, 1988; 1989). While not reporting hypotonicity-induced pH_i changes, elegant studies using tight epithelia have shown that pH_i regulates basolateral K^+ channels (Harvey, Thomas & Ehrenfeld; Harvey & Ehrenfeld, 1988*a*,*b*). Our current results are in accord with studies using HIV-gp120 in astrocytes which demonstrated that addition of gp120 stimulated amiloride-sensitive Na^+/H^+ exchange (Benos et al., 1994) and then Na^+-dependent alkalinization; a large conductance apamin-sensitive K^+ channel was activated in these studies (Bubien, Benveniste & Benos, 1995).

We conclude that there is a causal relationship between the alkalinization of pH_i, mobilization of Ca^{2+} and the activation of CTX-sensitive K^+ channels required for volume regulation in jejunal villus cells exposed to 0.93 × isotonic medium. Because the resulting modest volume increase mimics the size these cells swell during Na^+-solute absorption, which in turn triggers a distinct CTX-sensitive RVD response, we suggest there is a

physiological size increase which is distinctive to Na^+-absorbing villus epithelial cells. The magnitude of epithelial cell swelling is a key determinant of the signal transduction responsible for activating the K^+ channels required for subsequent volume regulation.

We gratefully thank P. Lembessis and A. Stryker for technical assistance. This work was supported by an operating grant from the Medical Research Council of Canada.

References

Andersen, H.K., Urbach, V., van Kerkhove, E., Prosser, E., Harvey, B.J. 1995. Maxi K^+ channels in the basolateral membrane of the exocrine frog skin gland regulated by intracellular calcium and pH. *Pfluegers Arch.* **431:**52–65

Altamirano, J., Brodwick, M.S., Alvarez-Leefmans, F.J. 1998. Regulatory volume decrease and intracellular Ca^{2+} in murine neuroblastoma cells studied with fluorescent probes. *J. Gen. Physiol.* **112:**145–160

Benos, D.J., Hahn, B.H., Bubien, J.K., Ghosh, S.K., Washburn, N.A., Chaikin, M.A., Shaw, G.M., Benveniste, E.N. 1994. Envelope glycoprotein gp120 of human immunodeficiency virus type 1 alters ion transport in astrocytes: Implications for AIDS dementia complex. *Proc. Natl. Acad. Sci. USA* **91:**494–498

Bubien, J.K., Benveniste, E.N., Benos, D.J. 1995. HIV-gp120 activates large-conductance apamin-sensitive potassium channels in rat astrocytes. *Am. J. Physiol.* **268:**C1440–C1449

Christensen, O. 1987. Mediation of cell volume regulation by Ca^{2+} influx through stretch-activated channels. *Nature* **330:**66–68

Christensen, O., Zeuthen, T. 1987. Maxi K^+ channels in leaky epithelia are regulated by intracellular Ca^{2+}, pH and membrane potential. *Pfluegers Arch.* **408:**249–259

Cook, D.L., Ikeuchi, M., Fujimoto, W.Y. 1984. Lowering pH_i inhibits Ca^{2+}-activated K^+ channels in pancreas β-cells. *Nature* **311:**269–271

Copello, J., Segal, Y., Reuss, L. 1991. Cytosolic pH regulates maxi K^+ channels in *Necturus* gall-bladder epithelial cells. *J. Physiol.* **434:**577–590

Cornejo, M., Guggino, S.F., Guggino, W.B. 1990. Ca^{2+}-activated K^+ channels from cultured renal medulla thick ascending limb cells: Effect of pH. *J. Membrane Biol.* **110:**49–55

Deutsch, C., Lee, S.C. 1988. Cell volume regulation in lymphocytes. *Renal Physiol. Biochem.* **3–5:**260–276

Deutsch, C., Lee, S.C. 1989. Modulation of K^+ currents in human lymphocytes by pH. *J. Physiol.* **413:**399–413

Foskett, J.K. 1994. The role of calcium in the control of volume regulatory transport pathways. *In:* Cellular and Molecular Physiology of Cell Volume Regulation, K. Strange, editor. pp. 259–278. CRC Press, Boca Raton

Garcia, M.L., Knaus, H-G., Munujos, P., Slaughter, R.S., Kaczorowski, G.J. 1995. Charybdotoxin and its effect on potassium channels. *Am. J. Physiol.* **269:**C1–C10

Grinstein, S., Rothstein, A., Cohen, S. 1985. Mechanism of osmotic activation of Na^+/H^+ exchange in rat thymic lymphocytes. *J. Gen. Physiol.* **85:**765–787

Grissmer, S., Lewis, R.S., Cahalan, M.D. 1992. Ca^{2+}-activated K^+ channels in human leukemic T cells. *J. Gen. Physiol.* **99:**63–84

Grissmer, S., Nguyen, A., Cahalan, M.D. 1993. Calcium activated potassium channels in resting and activated human T lymphocytes. *J. Gen. Physiol.* **102:**601–630

Harvey, B.J., Thomas, R., Ehrenfeld, J. 1988. Intracellular pH controls cell membrane Na^+ and K^+ conductances and transport in frog skin epithelium. *J. Gen. Physiol.* **92:**767–791

Harvey, B.J., Ehrenfeld, J. 1988. Role of Na^+/H^+ in the control of intracellular pH and cell membrane conductances in frog skin epithelium. *J. Gen. Physiol.* **92:**793–810

Hazama, A., Okada, Y. 1990. Involvement of Ca^{2+}-induced Ca^{2+} release in the volume regulation of human epithelial cells exposed to a hypotonic medium. *Biochem. Biophys. Res. Commun.* **167:**287–293

Kaibara, M., Mitarai, S., Yano, K., Kameyama, M. 1994. Involvement of Na^+/H^+ antiporter in regulation of L-type Ca^{2+} channel current by angiotensin II in rabbit ventricular myocytes. *Circulation Res.* **75:**1121–1125

Klaerke, D.A., Weiner, H., Zenthen, T., Jorgensen, P.L. 1993. Ca^{2+} activation and pH dependence of a maxi K^+ channel from rabbit distal colon epithelium. *J. Membrane Biol.* **136:**9–21

Klöckner, U., Isenberg, G. 1994. Intracellular pH modulates the availability of vascular L-type Ca^{2+} channels. *J. Gen. Physiol.* **103:**647–663

Lang, F., Busch, G.L., Ritter, M., Völkl, H., Waldegger, S., Gulbins, E., Häussinger, D. 1998. Functional significance of cell volume regulatory mechanisms. *Physiol. Rev.* **78:**247–306

Latorre, R., Oberhauser, A., Labarca, P., Alvarez, O. 1989. Varieties of calcium-activated potassium channels. *Annu. Rev. Physiol.* **51:**385–399

Laurido, C., Candia, S., Wolff, D., Latorre, R. 1990. Proton modulation of a Ca^{2+}-activated K^+ channel from rat skeletal muscle incorporated in planar bilayers. *J. Gen. Physiol.* **98:**1025–1042

Lu, L., Markakis, D., Guggino, W.B. 1993. Identification and regulation of whole-cell Cl^- and Ca^{2+}-activated K^+ currents in cultured medullary thick ascending limb cells. *J. Membrane Biol.* **135:**187–189

MacKinnon, R., Miller, C. 1988. Mechanism of charybdotoxin block of the high-conductance, Ca^{2+}-activated K^+ channel. *J. Gen. Physiol.* **91:**335–349

MacLeod, R.J., Hamilton, J.R. 1999. Ca^{2+}/calmodulin kinase II and decreases in intracellular pH are required to activate K^+ channels after substantial swelling in villus epithelial cells. *J. Membrane Biol.* **172:**59–66

MacLeod, R.J., Hamilton, J.R. 1996. Activation of Na^+/H^+ exchange is required for regulatory volume decrease after modest 'physiological' volume increases in jejunal villus epithelial cells. *J. Biol. Chem.* **271:**23138–23145

MacLeod, R.J., Lembessis, P., Hamilton, J.R. 1992*a*. Differences in Ca^{2+}-mediation of hypotonic and Na^+-nutrient regulatory volume decrease in suspensions of jejunal enterocytes. *J. Membrane Biol.* **130:**23–31

MacLeod, R.J., Lembessis, P., Hamilton, J.R. 1992*b*. Effect of protein kinase C inhibitors on Cl^- conductance required for volume regulation after L-alanine cotransport. *Am. J. Physiol.* **262:**C950–C955

MacLeod, R.J., Lembessis, P., Hamilton, J.R. 1992*c*. Effect of osmotic swelling on K^+ conductance in jejunal crypt epithelial cells. *Am. J. Physiol.* **262:**G1021–G1026

MacLeod, R.J., Hamilton, J.R. 1991*a*. Volume regulation initiated by Na^+-nutrient cotransport in isolated mammalian villus enterocytes. *Am. J. Physiol.* **260:**G26–G33

MacLeod, R.J., Hamilton, J.R. 1991*b*. Separate K^+ and Cl^- transport pathways are activated for regulatory volume decrease in jejunal villus cells. *Am. J. Physiol.* **260:**G405–G415

MacLeod, R.J., Hamilton, J.R. 1990. Regulatory volume increase in mammalian jejunal villus cells is due to bumetanide-sensitive $NaKCl_2$ cotransport. *Am. J. Physiol.* **258:**G665–G674

McCarty, N.A., O'Neil, R.G. 1992. calcium signaling in cell volume regulation. *Physiol. Rev.* **72:**1037–1061

McCarty, N., O'Neil, R.G. 1991. Calcium-dependent control of volume regulation in renal proximal tubule cells: 1. Swell-activated Ca^{2+} entry and release. *J. Membrane Biol.* **123:**149–160

Missiaen, L., DeSmedt, H., Parys, J.B., Sienaert, J., Vanlingen, S., Droogmans, G., Nilius, B., Casteels, R. 1996. Hypotonically induced calcium release from intracellular calcium stores. *J. Biol. Chem.* **271:**4601–4604

Park, K-P., Beck, J.S., Douglas, I.J., Brown, P.D. 1994. Ca^{2+}-activated K^+ channels are involved in regulatory volume decrease in acinar cells isolated from rat lacrimal gland. *J. Membrane Biol.* **141:**193–201

Powell, W.S., Gravel, S., MacLeod, R.J., Mills, E., Hashefi, M. 1993. Stimulation of human neutrophils by 5-oxo-6,8,11,14 eicosatetraenoic acid by a mechanism independent of the leukotriene B_4 receptor. *J. Biol. Chem.* **268:**9280–9286

Reinhart, P.H., Chung, S., Martin, B.L., Brantigan, D.L., Levitan, I. 1991. Modulation of calcium-activated K^+ channels from rat brain by protein kinase A and phosphatase 2A. *J. Neurosci.* **11:**1627–1635

Ross, P.E., Cahalan, M.D. 1995. Ca^{2+} influx pathways mediated by swelling or stores depletion in mouse thymocytes. *J. Gen. Physiol.* **106:**415–444

Rothermel, J.D., Botelho, L.H., Parker, D. 1988. A mechanistic and kinetic analysis of the diastereoisomers of adenosine 3′,5′-(cyclic) phosphorothioate. *Biochem. J.* **251:**757–762

Shmigol, A., Smith, R.D., Taggart, M.J., Wray, S., Eisner, D.A. 1995. Changes of pH affect calcium currents but not outward potassium currents in rat myometrial cells. *Pfluegers Arch.* **431:**135–137

Suzuki, M., Kawahara, K., Ogawa, A., Morita, T., Kawaguchi, S., Kurihara, S., Sakai, O. 1990. $[Ca^{2+}]_i$ rises via G protein during regulatory volume decrease in rabbit proximal tubule cells. *Am. J. Physiol.* **258:**F690–F696

Tanc, M., Congar, P., Poncet, V., Merot, J., Vita, C., Ponjeol, P. 1993. Toxic pharmacology of the large-conductance Ca^{2+}-activated K^+ channel in the apical membrane of rabbit proximal convoluted tubule in primary culture. *Pfluegers Arch.* **425:**126–133

Yamakage, M., Lindeman, K., Hirshman, C., Croxton, T. 1995. Intracellular pH regulates voltage-dependent Ca^{2+} channels in porcine tracheal smooth muscle cells. *Am. J. Physiol.* **268:**L642–L646