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Ca^{2+}/Calmodulin Kinase II and Decreases in Intracellular pH are Required to Activate K^+ Channels After Substantial Swelling in Villus Epithelial Cells

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Abstract. To assess the activation of the charybdotoxin-insensitive K^+ channel responsible for Regulatory Volume Decrease (RVD) after substantial volume increases, we measured intracellular pH (pH_i), intracellular calcium ($[Ca^{2+}]_i$) and inhibitors of kinases and phosphoprotein phosphatases in guinea pig jejunal villus enterocytes in response to volume changes. Fluorescence spectroscopy was used to measure pH_i and $[Ca^{2+}]_i$ of cells in suspension, loaded with 2,7,bis-carboxyethyl-5-6-carboxyfluorescein and Indo-1, respectively, and cell volume was assessed using electronic cell sizing. A modest 7% volume increase or substantial 15 to 20% volume increase caused $[Ca^{2+}]_i$ to increase proportionately but the 7% increase caused alkalinization while the larger increases resulted in acidification of $\simeq$0.14 pH units. Following a 15% volume increase, 1-N-0-bis (5-isoquinoline-sulfonyl)-N-methyl-L-4-phenyl-piperazine (KN-62, 50 μM), an inhibitor of Ca^{2+}/calmodulin kinase II, blocked RVD. Gramicidin (0.5 μM) bypassed this inhibition suggesting that the K^+ channel had been affected by the KN-62. RVD after a modest 7% volume increase was not influenced by KN-62 unless the cell was acidified. Okadaic acid, an inhibitor of phosphoprotein phosphatases 1 and 2A, accelerated RVD after a 20% volume increase; inhibition of RVD generated by increasing the K^+ gradient was bypassed by okadaic acid. Tyrosine kinase inhibitor, genistein (100 μM) had no effect on RVD after 20% volume increases. We conclude that activation of charybdotoxin-insensitive K^+ channels utilized for RVD after substantial (>7%) 'nonphysiological' volume increases requires phosphorylation mediated by Ca^{2+}/calmodulin kinase II and that increases in cytosolic acidification rather than larger increases in $[Ca^{2+}]_i$ are a critical determinant of this activation.

Key words: RVD — Ca^{2+} — pH_i — Ca^{2+}/calmodulin kinase II — Villus epithelial cell — K^+ channels

Introduction

Swelling of a jejunal villus epithelial cell stimulates the activation of K^+ and Cl^- channels which generate a Regulatory Volume Decrease (RVD) (MacLeod & Hamilton, 1991*a*,*b*); the signal transduction responsible for activating the K^+ channels is dependent on the extent of the volume increase which occurs (MacLeod & Hamilton, 1996, 1999). Following 'standard' hypotonic dilution (0.70 × isotonic) routinely used in volume regulation protocols (reviewed in Hoffmann & Dunham, 1995; Strange et al., 1996; Lang et al., 1998), villus cells swell $\simeq$15%; and charybdotoxin, an inhibitor of maxi-K^+ channels, does not block the RVD which is triggered by this substantial swelling. This response contrasts with the response to volume increases of 5 to 7% occurring because of Na^+-solute absorption, or in response to a modest hypotonic dilution. The K^+ channel utilized after 5 to 7% volume increases is charybdotoxin (CTX)-sensitive and requires increases in both intracellular pH (pH_i) and calcium $[Ca^{2+}]_i$ for activation (MacLeod et al., 1992*a*,*b;* MacLeod & Hamilton, 1996, 1999). To characterize the signal transduction responsible for activating the CTX-insensitive K^+ channels required for K^+ loss after substantial volume increases, we measured changes in pH_i and $[Ca^{2+}]_i$ following 'standard' 0.70 × isotonic hypotonic dilutions.

Because we had previously shown that calmodulin antagonists prevent activation of the CTX-insensitive K^+

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channel (MacLeod & Hamilton, 1992*b*) and phosphorylation can modulate the activity of some high conductance, Ca^{2+}-activated (maxi-K^+) channels (Levitan, 1994; Esguerra et al., 1994) we assessed villus cell volume responses to different kinase and phosphoprotein phosphatase inhibitors. After substantial volume increases, we found these inhibitors had a distinct effect on volume regulation which was dependent on the extent of intracellular acidification rather than $[Ca^{2+}]_i$. Our results support the concept that while the same osmolyte (K^+) is lost for volume regulation after modest or substantial volume increases in these epithelial cells, different signal transduction pathways for the activation of K^+ channels are utilized.

Materials and Methods

Villus Cell Isolation and Volume Determinations

Villus enterocytes were isolated from segments of adult male (200–300 g) guinea pig jejunum by mechanical vibration (MacLeod & Hamilton, 1990). Isolated cells were resuspended at 0.8 to 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 = 0.976) with direct measurements of cell water (MacLeod & Hamilton, 1991*b*). Relative cell volume was determined as the ratio of cell volume under experimental conditions to volume under basal, isotonic conditions.

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

pH_i was determined fluorometrically using BCECF, as previously described (MacLeod & Hamilton, 1996, 1999). $[Ca^{2+}]_i$ was measured fluorometrically using Indo-1, exactly as described in a separate study (MacLeod & Hamilton, 1999).

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_20). Na^+ propionate medium contained (in mM): 110 NaCl, and 30 Na^+ propionate, as well as the other components of the Na^+ medium. K^+ medium containing 10 and 20 mM K had NaCl isoosmotically replaced by KCl. Na^+-free medium was prepared by isoosmotically replacing NaCl with the chloride salt of N-methyl-D-glucamine ($NMDG^+$).

Reagents

Okadaic acid was purchased from Moana Bioproducts (Honolulu, HI). KN-62 and 5-N-methyl(-N-isobutyl)-amiloride (MIA) were from Research Biochemicals (Natick, MA) and $NMDG^+$ from Aldrich (Milwaukee, WI). Gramicidin and genistein were from Sigma Chemical (St. Louis, MO). The acetoxymethyl ester of 2′7′ bis (carboxyethyl)5-(6)-carboxyfluorescein (BCECF) and Indo-1 were obtained from Molecular Probes (Eugene, OR). RPMI-1640 (10×) medium was from GIBCO/BRL (Burlington, Ont).

Statistics

Data are reported as means ± SE of five to twelve experiments performed in duplicate. Significance of differences in means were determined either by Student's *t*-test or by ANOVA.

Results

Effect of Inhibitors of Ca^{2+}-Calmodulin Kinase II, Phosphoprotein Phosphatases and Tyrosine Kinases on RVD of Jejunal Villus Enterocytes Suspended in Hypotonic Media

The inhibitor of Ca^{2+}-calmodulin kinase II, KN-62 (1-N-0-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl-4-phenylpiperazine) (50 μM) inhibited RVD after a substantial hypotonic challenge (0.70 × isotonic) but not after a more modest challenge (0.93 × isotonic) designed to duplicate the extent of volume increase during D-glucose or L-alanine absorption (Fig. 1*a*, and *b*). In Fig. 1*a*, gramicidin (0.5 μM) is seen to allow a complete bypass of this inhibition of RVD by KN-62 after 0.70 × isotonic dilution (final relative vol.: 0.95 ± 0.02 *vs.* 1.14 ± 0.01, $P < 0.001$, $n = 5$) suggesting that it is the volume-sensitive K^+ channel which is the target of Ca^{2+}-calmodulin kinase II.

If the volume-sensitive K^+ channel is activated by phosphorylation, via Ca^{2+}-calmodulin kinase II, inhibitors of phosphoprotein phosphatases should affect volume regulation. Okadaic acid (1 μM), a cell permeant inhibitor of phosphoprotein phosphatases 1 and 2A (Hardie et al., 1991) when added to these cells after a 0.70 × isotonic dilution in Na^+-free medium (Fig. 2), accelerated the initial rate of regulatory volume decrease (% vol. decrease · $cell^{-1}$ · min^{-1}: 5.0 ± 0.5 *vs.* 2.5 ± 1.0, $P < 0.05$, $n = 6$). The final relative volume of the cells treated with okadaic acid was less compared with untreated controls (0.96 ± 0.01 *vs.* 1.03 ± 0.01, $P < 0.05$, $n = 6$). To further characterize the influence of the phosphatase inhibitor on the K^+ channel, we altered extracellular K^+ concentrations and determined if okadaic acid influenced volume regulation (Fig. 3). In hypotonic medium, after a 0.5 × isotonic dilution when $[K^+]_0$ was 20 mM, final relative cell volume was greater compared than that of control cells suspended in $[K^+]_0$ of 3 mM (1.15 ± 0.02 *vs.* 1.00 ± 0.01, $P < 0.05$, $n = 5$, Fig. 3*A*). Okadaic acid (1 μM) added after this 0.5 × isotonic dilution accelerated their initial rate of shrinkage (% vol. decrease cell-1 · min^{-1}: 3.5 ± 0.2 *vs.* 1.0 ± 0.5, $P < 0.05$, $n = 5$). The cells treated with okadaic acid also reached a smaller relative volume compared with untreated controls (final rel. vol.: 1.04 ± 0.02 *vs.* 1.15 ± 0.02, $P < 0.05$). When the extracellular K^+ was 10 mM, addition of okadaic acid had no effect on the initial rate of cell shrinkage, but caused the cells to reach a smaller final relative volume

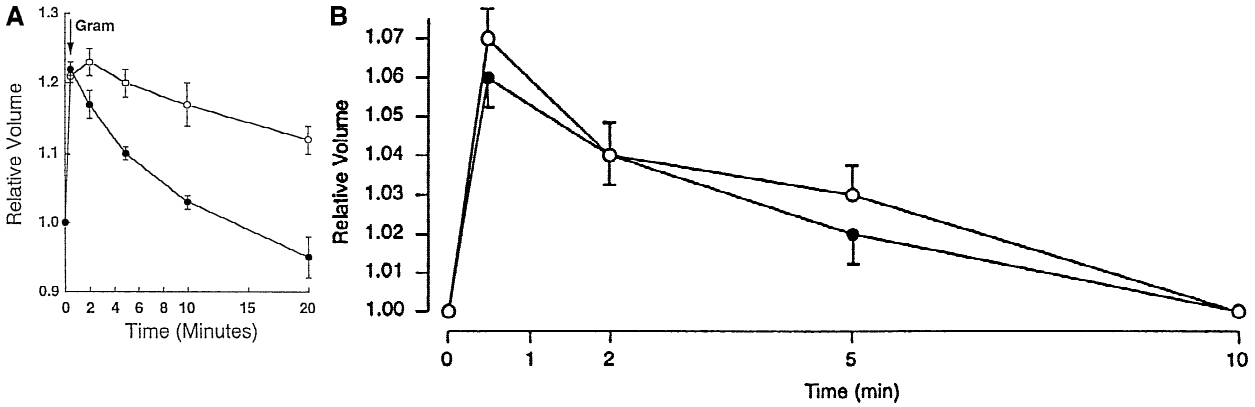

Fig. 1. Effect of 1-[N-0-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl-4-phenyl-piperazine (KN-62) on RVD. (*A*) ○, KN-62 (50 μM), 0.7 × isotonic; ●, gramicidin (0.5 μM) + KN-62, 0.7 × isotonic. (*B*) ●, KN-62, 0.93 × isotonic; ○, 0.93 × isotonic. $n = 5$. Volume measured electronically, expressed relative to isotonic control.

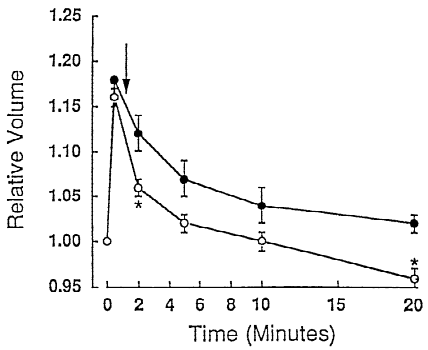

Fig. 2. Effect of okadaic acid A on RVD. ○, okadaic acid (1 μM), 0.7 × isotonic; ●, 0.7 × isotonic. Experiment performed in Na^+-free medium. $*P < 0.05$. $n = 5$.

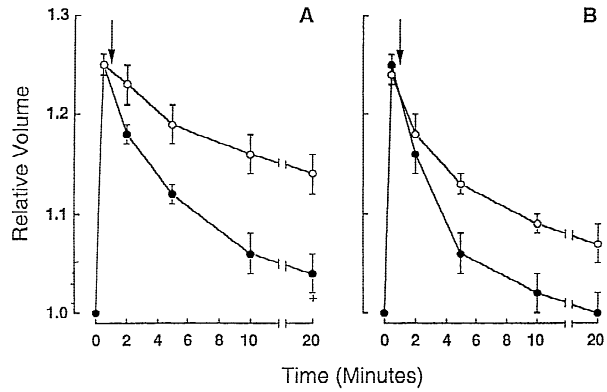

Fig. 3. Okadaic acid bypasses inhibition of RVD by extracellular K^+. (*A*) ○, 20 mM K^+, 0.5 × isotonic; ●, okadaic acid (1 μM) + 20 mM K^+, 0.5 × isotonic. (*B*) ○, 10 mM K^+, 0.5 × isotonic; ●, okadaic acid (1 μM) + 10 mM K^+, 0.5 × isotonic. $n = 5$. Arrow indicates okadaic acid addition.

(Fig. 3*B*). The final relative volume of these cells was less than untreated controls in 10 mM K^+ (1.00 ± 0.02 *vs.* 1.07 ± 0.02, $P < 0.05$, $n = 5$). Together, these data suggested that the activity of the KN-62-sensitive K^+ channel required for RVD after substantial volume increases may be modulated by an inhibitor of phosphoprotein phosphatases.

The tyrosine kinase inhibitor, genistein (100 μM) had no effect on the rate or extent of RVD after suspension of the cells in 0.70 × isotonic medium. There was no difference in the rate or extent of RVD in the presence of genistein compared with its absence (% vol. decrease: 17 ± 1 *vs.* 16 ± 1, $n = 4$). Comparable results were observed using 200 μM genistein.

Intracellular Calcium ($[Ca^{2+}]_i$) and Intracellular pH (pH_i) Responses of Villus Enterocytes to Different Degrees of Hypotonic Dilution

To determine whether the initial transient increase in $[Ca^{2+}]_i$ or the net change in pH_i were related to the activation of the KN-62-sensitive K^+ channel required for RVD after substantial volume increases, we first measured changes in $[Ca^{2+}]_i$ (Fig. 4) and pH_i (Fig. 5) of villus cells after different amounts of hypotonic dilution. The resting level of $[Ca^{2+}]_i$ in villus cells suspended in isotonic Na^+ medium was 144 ± 9 nM, $n = 14$. Each amount of hypotonic dilution (7 to 50%) caused an initial transient increase of $[Ca^{2+}]_i$ that was followed by a decline to a level above resting $[Ca^{2+}]_i$ (Fig. 4*A–D*). The initial transient increase of $[Ca^{2+}]_i$ was proportionate to the degree of hypotonic dilution (Table). These results suggested to us that the larger the villus cells swelled, the initial increase in $[Ca^{2+}]_i$ was proportionately greater.

The resting pH_i of the villus cells in isotonic, nominally HCO_3-free medium was 7.39 ± 0.05 pH units, $n = 21$. Figure 5 illustrates the changes in pH_i of these cells in suspension to different amounts (7 to 50%) of hypo-

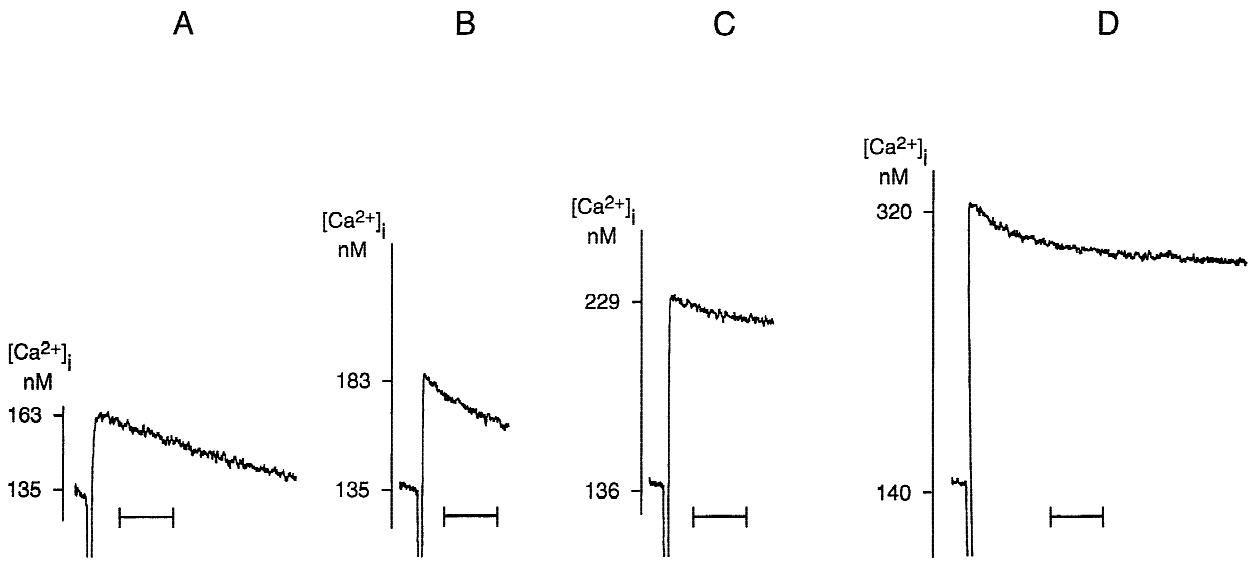

Fig. 4. Changes in $[Ca^{2+}]_i$ after modest (7%) or substantial (20–50%) hypotonic dilution. (*A*) 0.93 × isotonic, (*B*) 0.80 × isotonic, (*C*) 0.70 × isotonic, and (*D*) 0.50 × isotonic. Break in tracing is from opening compartment and making hypotonic dilution. Bar indicates 60 sec. Traces representative of one experiment. N of each treatment in the Table.

tonic dilution. As previously reported, a 0.93 × isotonic dilution, which generates a modest volume increase, caused a slight acidification that was followed by a robust alkalinization (Fig. 5*A*). Hypotonic dilutions >7% caused larger initial acidifications (Table) that were followed by continued acidifications (Figs. 5*B–D*). Thus, hypotonic dilutions >7% result in intracellular acidifications which are not followed by alkalinization.

Effect of Cytosolic Acidification of pH_i on KN-62 Sensitivity of RVD After Modest Volume Increases

Because substantial volume increases result in an initial acidification of ~0.140 pH units, we manipulated the resting pH_i of the villus cells to replicate this acidification and measured $[Ca^{2+}]_i$, pH_i and the response of cell volume to KN-62 after 0.93 × isotonic dilution (Fig. 6). Suspended for 15 min in isotonic Na^+ medium containing 30 mM Na propionate and 300 μM Zn^{2+} (to block H^+ conductance) the resting pH_i of the cells declined from 7.39 ± 0.05 to 7.25 ± 0.04 pH units ($P < 0.05$, $n = 15$). There was no difference in the volume of the propionate- and Zn^{2+}-treated cells compared with cells in regular Na^+ medium (1534 ± 35 *vs.* 1477 ± 48 fL^3, $P = 0.3631$, $n = 15$). A 0.93 × isotonic dilution of these cells resulted in a modest volume increase (rel. vol. 1.05 ± 0.01) that was followed by complete RVD (Fig. 6*A*). The initial transient $[Ca^{2+}]_i$ increase after hypotonic dilution was 19 ± 7 nM ($P < 0.05$, $n = 12$) above basal and remained sustained 15 ± 8 nM above this level (Fig. 6*B*). The pH_i acidified 0.050 ± 0.010 pH units and remained at 7.20 ± 0.01 pH units for 10 min (Fig. 6*C*). Under these conditions, RVD following this modest volume increase was prevented by KN-62 (50 μM). The final relative volume of cells treated with this inhibitor was larger than untreated controls (1.05 ± 0.01, $P < 0.001$, $n = 6$).

Discussion

In the current study we found that after a substantial volume increase, villus epithelial cells showed RVD due to K^+ channel activation which was blocked by an inhibitor of Ca^{2+}/calmodulin kinase II (CaMKII), accentuated by an inhibitor of phosphoprotein phosphatases and not influenced by an inhibitor of tyrosine kinase. The absence of a response to the tyrosine kinase inhibitor (Akiyama & Ogawara, 1991) genistein using freshly isolated villus enterocytes from mature guinea pig jejunum differ from the findings of others using the fetally derived intestine 407 line (Tilly et al., 1993, 1996). Various tyrosine kinase inhibitors either block volume-activated Cl^- current (Lepple-Wienhues et al., 1998; Voets et al., 1998) or had no effect on this current (Szücs et al., 1996; Gosling et al., 1995). However, following modest 'physiological' volume increase, the subsequent RVD, which was insensitive to the CaMKII inhibitor, became sensitive to this inhibitor if the pH_i of the villus cells was acidified to a degree (> 0.14 pH units) comparable to that observed with large volume increases. These larger volume increases caused greater transient increases in $[Ca^{2+}]_i$ than the modest 'physiological' vol-

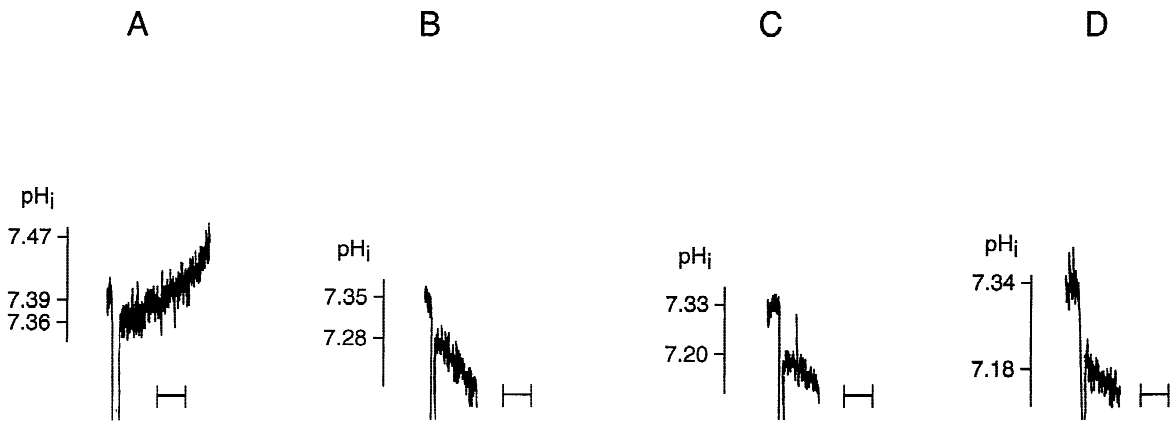

Fig. 5. Changes in pH_i after modest (7%) or substantial (20–50%) hypotonic dilution. (*A*) 0.93 × isotonic, (*B*) 0.80 × isotonic, (*C*) 0.70 × isotonic, and (*D*) 0.50 × isotonic. Tracings from a single experiment are illustrated. Bar indicates 60 sec. For *N* of each treatment *see* Table.

Table 1. Changes in $[Ca^{2+}]_i$ and pH_i after different degrees of hypotonic dilution

Hypotonic challenge	Initial $[Ca^{2+}]_i$ increase (nM)	Initial acidification (ΔpH_i, pH units)
0.93 × isotonic	33 ± 7 (6)	0.030 ± 0.010 (6)
0.80 × isotonic	59 ± 6 + (5)	0.070 ± 0.010 (6)
0.70 × isotonic	97 ± 10 (7)	0.125 ± 0.020 (6)
0.60 × isotonic	131 ± 5 (6)	*ND*
0.50 × isotonic	181 ± 12[a] (6)	0.150 ± 0.020[b] (14)

+$P < 0.05$ *vs.* 0.93 × isotonic; [a]$P < 0.05$ *vs.* 0.60 × isotonic; [b]$P < 0.05$ *vs.* 0.93 and 0.80 × isotonic. Data are means ± SE. Number of experiments in parentheses. ND, Not Done.

ume increase. Because the change in $[Ca^{2+}]_i$ in the acidified villus cells after a modest volume increase was not comparable to large volume increases but the change in pH_i was comparable, our results suggest that intracellular acidification, but not proportionately greater increases in $[Ca^{2+}]_i$, is a determinant of CaMKII sensitive K^+ channel activation required for RVD after large volume increases.

Previously we have shown that calmodulin antagonists W-7 and W-13 prevent RVD after substantial volume increases but they have no effect on RVD after modest 'physiological' volume increases caused by L-alanine absorption (MacLeod et al., 1992*b*). Because the cation ionophore gramicidin bypassed RVD inhibition by calmodulin antagonists, we conclude that the K^+ channel activated for RVD requires calmodulin. Our current experiments with KN-62, synthesized as a specific inhibitor of CaMKII (Hidaka et al., 1984; Tokomitsu et al., 1990; Tsumoda et al., 1992) extend this interpretation. Since the inhibitory effects of KN-62 on CaMKII could also be bypassed by providing a surrogate K^+ channel, our results indicate that it is K^+ and not Cl^- conductance which is influenced by CaMKII. Furthermore, the inhibition of the volume regulation caused by KN-62 was not due to this inhibitor's hydrophobicity since RVD after modest 'physiological' swellings proceeded normally in the presence of KN-62. In rat intestine CaMKII has been localized to the terminal web of the apical membrane of villus cells (Fukunaga et al., 1993). The demonstrated pharmacological sensitivity of RVD to KN-62 after large volume increases is strong evidence that CaMKII is required to activate the K^+ channel required for volume regulation.

In the current experiments, we found that a membrane permeant phospho-Ser/Thr phosphatase inhibitor, Okadaic acid (OA) accelerated the rate of RVD after a substantial volume increase as well as bypassed inhibition of volume changes that were rate-limited by the K^+ gradient. The dose dependency of OA strongly suggests the involvement of phosphorylation and a phospho-Ser/Thr phosphatase in regulating activation of the KN-62 sensitive K^+ channel (Shenolikar & Nairin, 1991). It has been shown that OA does not effect protein kinase C, A or CaM kinases at concentrations up to 5 μM (Bialojan & Takai, 1988; Cohen, 1989; Hardie et al., 1991). The effect of OA on NHE activity of resting lymphocytes (Bianchini et al., 1991) and NaK2Cl activity in duck erythrocytes (Pewitt et al., 1990; Palfrey, 1994) have demonstrated that constitutive phosphorylation of the antiporter or cotransporter were related to activity. Furthermore, OA has been used to alter the rates of activity of red cell KCl cotransport after hypotonic challenge suggesting that this cotransporter or a putative cofactor were regulated by net dephosphorylation (Jennings & Al-Rohil, 1990; Kaji & Tsuketani, 1991; Jennings & Schulz, 1991). In the current study, we found that when volume changes were rate-limited by the K^+ gradient, OA bypassed this inhibition of RVD. Therefore, because of the distinct effects of inhibitors of phosphoprotein phosphatases and CaMKII on volume regulation after substantial volume increases we conclude that phosphorylation me-

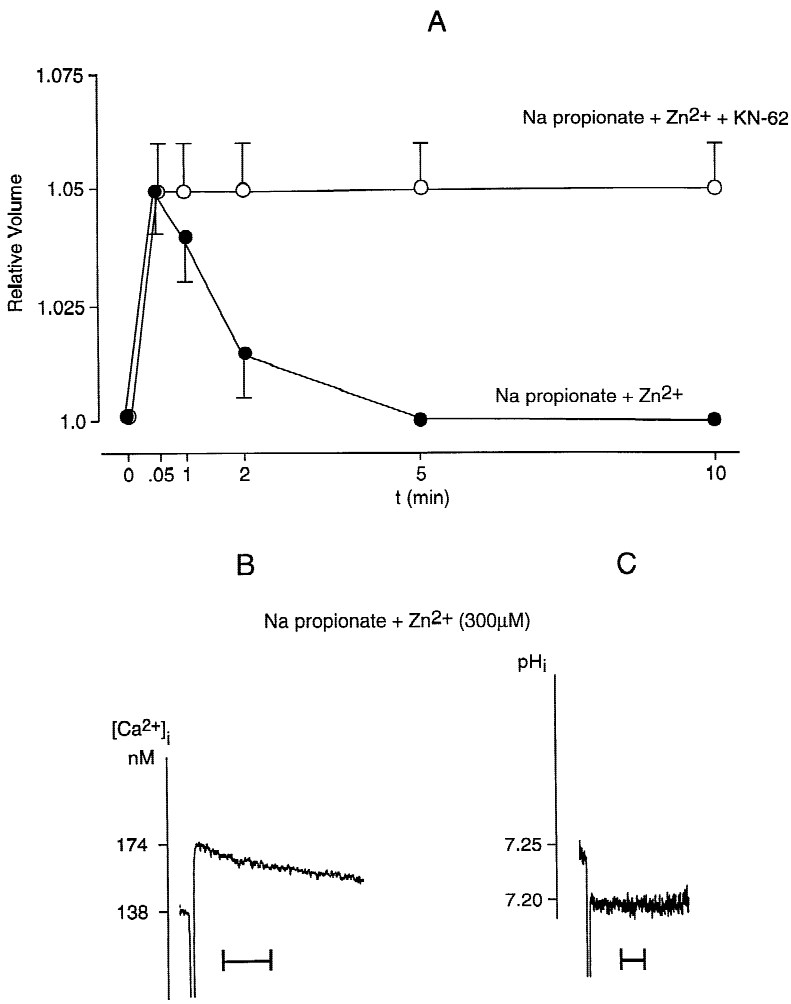

Fig. 6. Effect of intracellular acidification on RVD, $[Ca^{2+}]_i$ and pH_i after modest volume increase. (*A*) ○, 0.93 × isotonic in Na^+ medium containing 30 mM Na^+ propionate and 300 μM $ZnCl_2$; ●, 0.93 × isotonic, KN-62 (50 μM) in Na propionate and Zn^{2+} containing Na^+ medium. $n = 6$. (*B*) $[Ca^{2+}]_i$ in the Na^+ propionate, Zn^{2+} containing medium. (*C*) pH_i changes after 0.93 × isotonic treatment in Na^+ propionate Zn^{2+} containing medium. Tracings representative of 12 experiments performed in duplicate, results of one experiment illustrated.

diated by CaMKII is required to activate the KN-62 sensitive K^+ channel required for RVD.

Both modest (5 to 7%) and substantial (15 to 20%) volume increases resulted in proportionate increases in intracellular calcium but intracellular alkalinization occurred with the former while acidification occurred with the latter. Increases in $[Ca^{2+}]_i$ after villus cell swelling were comparable to changes in $[Ca^{2+}]_i$ recorded after hypotonic dilution in other cell types (Rothstein & Mack, 1989; McCarty & O'Neil, 1992). Because KN-62 will only bind CaMKII and not calmodulin alone (Tokumitsu et al., 1990) our current findings suggest a cascade where an increase in $[Ca^{2+}]_i$ activates calmodulin and the Ca^{2+}-calmodulin then activates CaMKII. Acidifying villus cells to replicate the pH_i response to a substantial volume increase and then initiating modest 'physiological' cell swelling attenuated the initial $[Ca^{2+}]_i$ increase but allowed activation of the KN-62 sensitive K^+ channel. Thus, while an increase in $[Ca^{2+}]_i$ was necessary for the CaMKII activation, acidification of pH_i appeared to be a key determinant of the KN-62 sensitive K^+ channel activation.

The usual experimental protocols to acidify epithelial cells such as withdrawal of extracellular Na^+ or addition of an amiloride derivative could not be used in the current studies. The Na^+ withdrawal protocol could not be used because after Na^+ withdrawal the villus cells aggressively recover from the subsequent acidification by activating a V-type H^+-ATPase and a Zn^{2+}-sensitive H^+ conductance (MacLeod & Hamilton, 1996, 1997*a*). Methyl-isobutyl-amiloride addition causes an acidification of only 0.08 pH units, half the amount caused by 15% volume increases and Na^+ propionate addition to these cells resulted in a maximal acidification of 0.10 pH units, a level insufficient to permit RVD to progress (MacLeod & Hamilton, 1996). Therefore we empirically defined conditions (preincubation with 30 mM Na^+ propionate) which caused a comparable net acidification to that seen after substantial volume increases while blocking pH_i recovery with Zn^{2+} (MacLeod & Hamilton, 1997*b*); under these conditions, RVD was complete. There was no difference in the volume of these cells compared with cells in propionate-free medium. Consequently, we assume that if propionate had acti-

vated NHE isoforms to cause cell swelling, then homeostatic volume mechanisms like NaK-ATPase or NaK2Cl cotransport efflux may have compensated for any volume changes (MacLeod & Hamilton, 1990).

The sources of the acidification during substantial volume increases are unknown, but it has been shown that a 15% volume increase inhibits the NHE-1 isoform (MacLeod & Hamilton, 1996). Others have reported inhibition of NHE activity (Green et al., 1988), increased metabolic activity (Livne, Grinstein & Rothstein, 1987) as well as conductive OH^- efflux (Star et al., 1992) as potential determinants of net acidification during substantial volume increases. When taken together, our observations lend further support to the concept that net acidification of pH_i is a critical determinant of the activation of the KN-62 sensitive K^+ channel for volume regulation.

Two different signal transduction pathways exist for K^+ channel activation required for RVD. Each pathway is determined by the direction and extent of the change in pH_i occurring because of swelling. Modest (5 to 7%) or 'physiological' volume increases which duplicate the size these cells swell when they absorb L-alanine or D-glucose together with sodium, activate the basolateral NHE-1 isoform of NHE which results in an alkalinization (MacLeod & Hamilton, 1996, 1999). This cytosolic alkalinization then increases cell Ca^{2+}; and both increases in cytosolic Ca^{2+} and pH_i allow activation of the CTX-sensitive K^+ channel. It is unclear how this activation occurs. It is not through PKA, PKC, or CaMKII. However, a rise in Ca^{2+} alone is insufficient for activation. The requirement of intracellular alkalinization for activation may be to stimulate an appropriate kinase, as others have shown that cytosolic alkalinization, independent of volume changes, could substantially increase activities of stress and p38 mitogen-activated kinases (Shrode et al., 1997). Nevertheless, both an increase in Ca^{2+} and in pH_i are required to activate the CTX-sensitive K^+ channel for RVD. In contrast, activation of the KN-62 sensitive K^+ channel after substantial volume increases requires both cytosolic acidification and CaMKII activity. We speculate that the increase in $[Ca^{2+}]_i$ is required to activate the kinase and that acidification facilitates channel activation via this kinase. Cytosolic acidification will inhibit the CTX-sensitive K^+ channel. Indeed, alkalinizing cells after substantial volume increases causes CTX-sensitive volume changes, and acidifying cells after modest 'physiological' volume increases results in KN-62 sensitive volume changes. Clearly, these pH_i changes could explain differing inhibitor sensitivities when cells are swollen different amounts. Many volume regulatory studies presume that the same signal transduction pathways are responsible for activating osmolyte loss, irrespective of the extent a cell initially swells (Hoffmann & Dunham, 1995; Strange et al., 1996; Lang et al., 1998), for example, swelling-activated Ca^{2+} influx in thymocytes and Cl^- channel activation in lymphocytes is an 'all-or-none' response, while in HL-60 cells volume-sensitive Cl^- channel activation is graded to increasing hypotonicity (Ross & Cahalan, 1995; Hallows & Knauf, 1994; Sarkadi, Mack & Rothstein, 1984). However, in villus cells, the changes in pH_i occurring because of different magnitudes of swelling together with the differential sensitivity of RVD to calmodulin antagonists/kinase inhibitors strongly suggests that different signal transduction pathways are responsible for K^+ channel activation for volume regulation.

We conclude that two different signal transduction pathways exist for K^+ channel activation required for RVD in villus epithelial cells. When villus cells swell to amounts greater than that observed during Na^+-solute absorption, the resulting intracellular acidification facilitates CaMKII to activate a CTX-insensitive K^+ channel. The direction and extent of the change in pH_i that occurs is strictly dictated by the size these cells swell. Consequently, the size an epithelial cell swells is a determinant of the signal transduction responsible for activating the K^+ channel required for volume regulation.

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