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Differences in Ca^{2+}-Mediation of Hypotonic and Na^+-Nutrient Regulatory Volume Decrease in Suspensions of Jejunal Enterocytes

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Summary. We determined differences in the Ca^{2+} signalling of K^+ and Cl^- conductances required for Regulatory Volume Decrease (RVD) in jejunal villus enterocytes passively swollen (0.5 or 0.95 · isotonic) compared with swelling because of the absorption of D-glucose (D-Glc) or L-Alanine (L-Ala). Cell volume was measured using electronic cell sizing. In nominally Ca^{2+}-free medium containing EGTA (100 μM) RVD after 0.5 or 0.95 · isotonic challenge was prevented. L-Ala swelling and subsequent RVD was influenced in Ca^{2+}-free medium. Villus cells were incubated with 10 μM of the acetomethoxy derivative of 1,2.bis (2-aminophenoxy) ethane N,N,N^1,N^1 tetracetic acid (BAPTA-AM) and RVD after 0.5 · isotonic swelling or L-Ala swelling was prevented. Niguldipine (0.1 μM), nifedipine (5 μM), diltiazem (100 μM), Ni^{2+}, and Co^{2+} (1 mM) all prevented hypotonic RVD but had no effect on RVD after L-Ala addition. Charybdotoxin (25 nM) a potent inhibitor of Ca^{2+}-activated K^+ channels, had no effect on hypotonic RVD but prevented RVD of villus cells swollen by D-Glc. We used the calmodulin antagonists, napthalene sulfonamide derivatives W-7 and W-13, to assess calmodulin activation of K^+ and Cl^- conductance in these two models. L-Ala swelling and subsequent RVD was not influenced by 25 μM W-7; hypotonic RVD was prevented by 25 μM W-7 or 100 μM W-13. The W-13 inhibition of RVD was by-passed with 0.5 μM gramicidin. Our data show that hypotonic RVD requires extracellular Ca^{2+} and that the K^+ conductance activated is not charybdotoxin sensitive but requires calmodulin. Na^+-nutrient RVD requires intracellular calcium mobilization to activate a charybdotoxin-sensitive K^+ conductance. The signalling for RVD after cell swelling because of transport of osmotically active Na^+ nutrient is different than the signalling for RVD after passive hypotonic cell swelling.

Key Words villus cells · Ca^{2+} signalling · Na^+-nutrient RVD

Introduction

Intestinal epithelial cells must coordinate ion transport pathways responsible for their volume regulation and homeostasis with those responsible for transcellular solute transport (Schultz & Hudson, 1986; MacLeod & Hamilton, 1990). Using an electronic sizing technique, we have shown that passive cell swelling in hypotonic medium or swelling induced by Na^+-nutrient addition elicits Regulatory Volume Decrease (RVD) due to the activation of K^+ and Cl^- conductive pathways (MacLeod & Hamilton, 1991*a*,*b*). We believe that major differences exist in the ionic pathways activated in these two models of RVD. This conclusion stems from our recent observation that the activation of Cl^- conductance for RVD after L-Alanine induced cell swelling was prevented by inhibitors of protein kinase C, while these inhibitors had no effect on RVD or ^{36}Cl efflux after hypotonic swelling (MacLeod, Lembessis & Hamilton, 1992).

Calcium is known to mediate RVD in both symmetrical and epithelial cells (Christensen, 1987; Pierce & Politis, 1990). Extracellular Ca^{2+} is required for passive, hypotonic RVD in urinary bladder (Davis & Finn, 1985, Wong & Chase, 1986), kidney proximal straight tubule (McCarty & O'Neil, 1990), and a kidney cell line (Rothstein & Mack, 1990). The experiments to be reported evaluate the role of calcium in mediating RVD following osmotic and Na^+-nutrient induced swelling.

Materials and Methods

Villus Cell Isolation

Segments of adult male (200–300 g) guinea pig jejunum were everted over metal spiral rods and vibrated in a Ca^{2+}-free phosphate buffered physiologically balanced salt solution exactly as previously described (MacLeod & Hamilton 1990). Isolated cells were collected by centrifugation at 50 × *g* for 5 min and resuspended at 0.8–1.5 × 10^6 cells per 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%, 3 hr after suspension in medium. After cell separation, the remaining jejunal tissue consisted

of intact villus cores and crypt epithelia but no remaining villus cells.

Electronic Cell Sizing

Cell volume was measured using a Coulter Counter (model Zm) with an attached Channelyzer (C-256) as previously described (MacLeod & Hamilton, 1990, 1991*a,b*). Cell volume measured electronically over a range of tonicities correlated positively ($r = 0.967$) with direct measurements of cell water (MacLeod & Hamilton, 1990). Relative cell volume was determined as the ratio of cell volume under study conditions to the volume under basal conditions in an isotonic medium immediately before challenge.

Solutions

Cell volume measurements were made using 30,000 cells/ml in a 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). Nifedipine, diltiazem, W-7, W-12, W-13 and gramicidin were added to cells from stock solutions in dimethylsulfoxide. Intracellular Ca^{2+} was buffered by incubating cells with 10 μM of acetomethoxy 1,2-bis (2-aminophenoxy)ethase-N,N,N^1,N^1 tetraacetic acid (BAPTA-AM) for 30 min in RPMI-1640 medium, then centrifuging cells and resuspending them in BAPTA-AM free medium.

Reagents

Nifedipine, diltiazem and A23187 were from Calbiochem (San Diego, CA). Charybdotoxin was from Receptor Research Chemicals (Cockeysville, MD). Niguldipine was from Research Biochemicals (Natick, MA). Gramicidin, W-7, W-12, W-13 and EGTA was from Sigma Chemical (St. Louis, MO), BAPTA-AM from Molecular Probes (Eugene, OR) and RPMI-1640 medium was from GIBCO/BRL (Burlington, Ont.).

Statistics

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

Results

Effect of Ca^{2+}-Free Medium and 1,2-bis(2-Aminophenoxy)Ethane-N,N,N^1,N^1-Tetraacetic Acid on RVD

We first studied the volume response of villus enterocytes subject to an osmotic challenge (0.5 or 0.95 × isotonic) or following the addition of L-Alanine (20 mM) in nominally Ca^{2+}-free medium containing 0.1 mM EGTA (Fig. 1). After suspension in hypotonic medium (0.5 × isotonic), the cells rapidly swelled, but the RVD occurring in controls was prevented in the nominally Ca^{2+}-free medium (Fig. 1*A*). Final relative volume of control cells was less than cells in Ca^{2+}-free, 0.1 mM EGTA medium (1.05 ± 0.01 *vs.* 1.18 ± 0.02, $P < 0.001$, $n = 8$); shrinkage in the absence of Ca^{2+}, as a percentage of original volume was much less than that of controls (7.0 ± 3.0% *vs.* 20.0 ± 1.0%, $P < 0.001$). This response to a hypotonic medium contrasted with the effect of Ca^{2+}-free, EGTA medium on the volume response to L-Alanine (Fig. 1*B*). After the addition of L-Ala (20 mM) cells increased their relative volume, but within 2 min RVD began and progressed, unaltered by the presence or absence of calcium (maximal *vs.* final relative volume: 1.04 ± 0.01 *vs.* 0.98 ± 0.01, $P < 0.001$) (Fig. 1*B*). The percentage of cell shrinkage after L-Ala addition did not differ between the Ca^{2+} and Ca^{2+}-free conditions (12.0 ± 2.0% *vs.* 11.0 ± 1.0%, $n = 20$). When cells were suspended in hypotonic medium (0.95 × isotonic) so that they attained a volume increase equal to that occurring after L-Ala addition, RVD was prevented in Ca^{2+}-free medium (Fig. 1*C*). At the conclusion of the experiment, the final relative volume of cells in Ca^{2+}-free, EGTA medium was greater than control cells (1.04 ± 0.01 *vs.* 1.0, $P < 0.001$).

We then studied the effect of buffering a putative increase in $[Ca^{2+}]_i$ on RVD by treating villus enterocytes with 1,2 bis (2-aminophenoxy)ethane N,N,$N^1$$N^1$-tetraacetic and (BAPTA-AM) (Fig. 2). Following hypotonic swelling in Ca^{2+}-containing medium, villus enterocytes that had been loaded with BAPTA-AM (10 μM) did not exercise RVD (Fig. 2*A*). The final relative volume of cells that were loaded with BAPTA-AM was larger than controls (1.17 ± 0.01 *vs.* 1.03 ± 0.01, $P < 0.001$). The amount of cell shrinkage in BAPTA-AM loaded cells was less compared with control cells (5.8 ± 0.7% *vs.* 18.5 ± 2.0%, $P < 0.001$). As illustrated in Fig. 2*B*, RVD after L-Ala was prevented in BAPTA loaded cells; the final relative volume of cells incubated with BAPTA-AM and swollen by L-Ala addition was greater than cells swollen by L-Ala addition that subsequently underwent RVD (1.04 ± 0.01 *vs.* 0.96 ± 0.01, $P < 0.005$).

Effect of Ca^{2+} Channel Blockers on Hypotonic and Na^+ Nutrient RVD

Nifedipine (5 μM) a dihydropyridine Ca^{2+} channel blocker prevented RVD in villus enterocytes suspended in 0.5 × isotonic medium (Fig. 3*A*). The

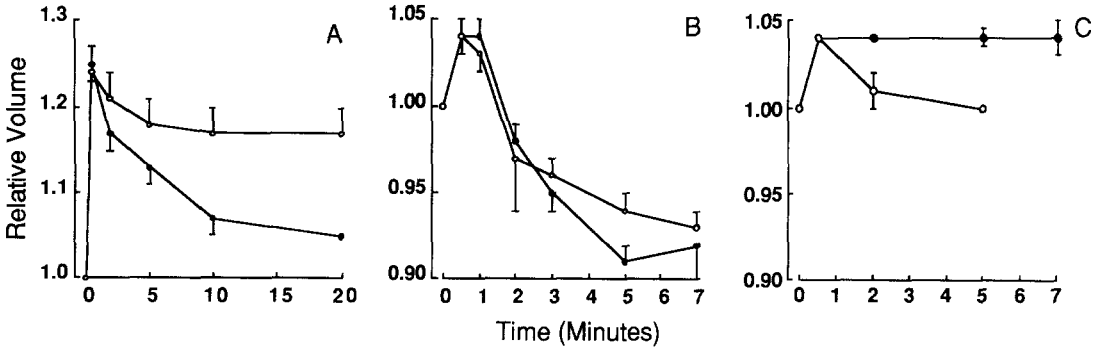


Fig. 1. Effect of extracellular Ca^{2+} chelation in RVD. (*A*) Hypotonic medium; ●, 0.5 × isotonic (1 mM Ca^{2+}); ○, 0.1 mM EGTA in Ca^{2+}-free medium; $n = 8$. (*B*) Volume changes after addition of 25 mM L-Ala ●, isotonic (1 mM Ca^{2+}); ○, 0.1 mM EGTA in Ca^{2+}-free medium; $n = 20$. (*C*) ○, 0.95 × isotonic in control; ●, 0.95 × isotonic in Ca^{2+}-free medium; $n = 5$. Volume was measured electronically, expressed relative to isotonic control.

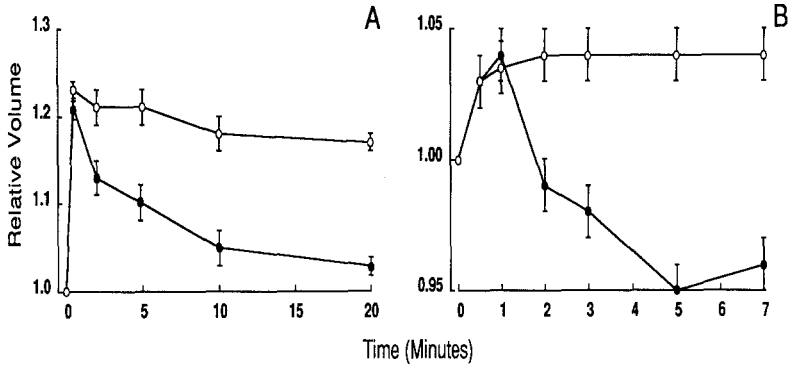


Fig. 2. Effect of intracellular Ca^{2+} buffering on RVD. Enterocytes were loaded with BAPTA/AM (10 μM) for 40 min, centrifuged and resuspended in RPMI as described in Materials and Methods. (*A*) Hypotonic medium: ●, 0.5 × isotonic; ○, BAPTA (10 μM) with 0.5 × isotonic; $n = 6$–8. (*B*) Volume changes after addition of 25 mM L-Alanine: ●, isotonic; ○, BAPTA (10 μM); $n = 6$ experiments performed in triplicate. Volume was measured electronically, expressed relative to isosmotic control.

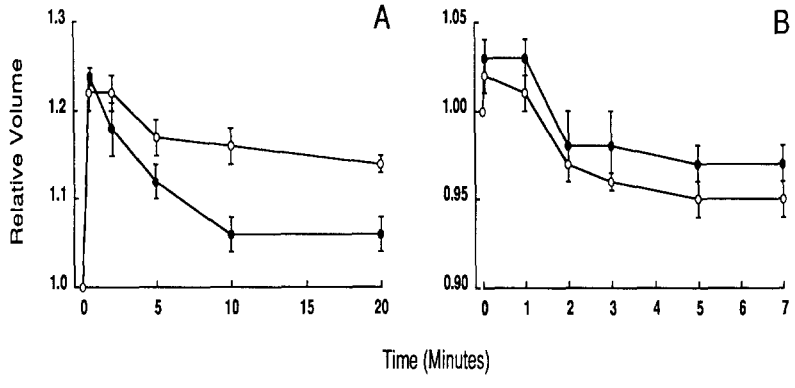


Fig. 3. Effect of dihydropyridine Ca^{2+} channel blocker, nifedipine, on RVD. (*A*) Hypotonic medium: ●, 0.5 × isotonic; ○, 0.5 × isotonic containing nifedipine (5 μM) (*B*) Volume changes after addition of 25 mM L-Alanine: Volume was measured electronically, expressed relative to isosmotic control.

final relative volume of cells in nifedipine-containing medium was greater than that of untreated controls (1.14 ± 0.01 *vs.* 1.06 ± 0.02, $P < 0.02$) and cell shrinkage was reduced by 5 μM nifedipine (7.8 ± 1.3% *vs.* 16.3 ± 1.9%, $P < 0.02$). However, nifedipine had no effect on volume changes initiated by L-Alanine (Fig. 3*B*). There was no difference in the amount of cell shrinkage in the presence of nifedipine compared with controls (6.3 ± 1.2% *vs.* 8.0 ± 1.2%). To determine whether the amount of cell swelling signalled sensitivity to nifedipine, we compared the effect of 0.9 × isotonic and 0.95 × isotonic medium in the presence of nifedipine on hypotonic RVD. In both cases, 0.9 × isotonic (final rel. vol. 1.06 ± 0.01, $P < 0.005$) and 0.95 × isotonic (final rel. vol. 1.03 ± 0.01, $P < 0.005$),

Table. Effect of Ca^{2+} channel blockers on regulatory volume decrease[a]

Inhibitor	Hypotonic	p[b]	L-Alanine	p
Control	21.0 ± 1.8	—	8.0 ± 0.8	—
Niguldipine 0.1 μM (n = 4)	9.7 ± 0.7	<0.01	8.0 ± 0.1	NS
Diltiazem 10 μM (n = 6)	13.0 ± 1.7	<0.01	ND	—
Diltiazem 100 μM (n = 6)	3.7 ± 0.3	<0.001	7.8 ± 0.7	NS
$NiCl_2$ 1 mM (n = 5)	3.8 ± 1.6	<0.001	7.8 ± 1.3	NS
$CoCl_2$ 1 mM (n = 5)	3.0 ± 0.8	<0.001	7.6 ± 0.4	NS

[a] Relative cell shrinkage (%) at 20 min (hypotonic) or 5 min (L-Alanine).
[b] p: Differences in means determined using Student's t test.
ND: Not determined.
NS: Not significant.

5 μM nifedipine prevented RVD following hypotonic swelling.

In further studies we compared the effect of the dihydropyridine Ca^{2+} channel blocker niguldipine, the benzothiazepine Ca^{2+} channel blocker diltiazem, and the inorganic Ca^{2+} channel blockers, Ni^{2+} and Co^{2+}, on these two types of RVD (Table). Niguldipine attenuated hypotonic RVD (0.1 μM, $P < 0.01$), but this concentration had no effect on RVD after swelling caused by L-Ala. In a dose-responsive manner, diltiazem prevented hypotonic RVD with a maximal effect at 100 μM ($P < 0.001$), but this concentration of diltiazem had no effect on RVD after cell swelling caused by L-Ala addition (Table). Similarly, both Ni^{2+} (1 mM, $P < 0.001$) and Co^{2+} (1 mM), $P < 0.001$) prevented hypotonic RVD but had no effect on RVD after cell swelling caused by L-Ala addition (Table).

Evidence that Diltiazem Inhibits Ca^{2+} Channels in Villus Enterocytes

Since diltiazem has been shown to inhibit K conductance in lymphocytes (Chandy et al., 1984) and since blocking K^+ conductance will prevent RVD in villus enterocytes (MacLeod & Hamilton, 1991*a,b*), we felt it was important to determine whether the diltiazem prevention of RVD we had observed was a direct result of Ca^{2+} channel inhibition. We postulated that if diltiazem were inhibiting Ca^{2+} channels, the calcium ionophore A23187 should bypass this inhibition, allowing Ca^{2+} influx and RVD to proceed; if this RVD inhibition were due to K^+ conductance inhibition, the calcium ionophore would not permit RVD to proceed in the presence of diltiazem since insufficient K^+ would be lost from the cells. Diltiazem (100 μM) prevented hypotonic RVD, but A23187 (2.5 μM) added to cells hypotonically swollen in the presence of diltiazem did allow RVD to proceed (Fig. 4*A*). The final relative volume of cells to which A23187 was added in medium containing diltiazem was less than control cells with diltiazem (0.99 ± 0.01 *vs.* 1.31 ± 0.02, $P < 0.001$). In contrast, an inhibitor of K^+ conductance, tetraethylammonium, (TEA, 20 mM) prevented RVD stimulated by A23187 (Fig. 4*B*). The final relative volume of cells in A23187 and TEA was not different from those in TEA alone (1.17 ± 0.02 *vs.* 1.17 ± 0.02, n = 8).

Effect of Charybdotoxin on Hypotonic and Na^+-Nutrient RVD

Previously, we have shown that A23187 (2.5 μM) caused a volume reduction of villus enterocytes suspended in isotonic Na^+-free medium due to Ca^{2+}-activated K^+ and Cl^- conductances (MacLeod et al., 1991). As shown in Fig. 5*A*, this A23187-stimulated volume reduction was inhibited by 25 nM charybdotoxin (CTX), a potent inhibitor of Ca^{2+}-activated K channels. The final relative volume of cells to which CTX was added was greater than controls (0.88 ± 0.01 *vs.* 0.77 ± 0.02, $P < 0.02$), suggesting that Ca^{2+}-activated K^+ channels were inhibited by 25 nM CTX. However, the charybdotoxin had no effect on hypotonic RVD; there were no differences in the final relative volume of cells compared with controls (1.06 ± 0.01 *vs.* 1.06 ± 0.01, n = 5) nor in cell shrinkage (21.0 ± 2.0% *vs.* 20.0 ± 1.2%, n = 5, Fig. 5*B*) between CTX-treated and control cells. This response contrasted with the effect of CTX on RVD following swelling induced by D-glucose (Fig. 5*C*) where we observed prevention of RVD. The final relative volume of cells in the presence of CTX was larger than controls (1.02 ± 0.01 *vs.* 0.92 ± 0.01, $P < 0.005$).

Effect of Calmodulin Antagonists on Hypotonic and Na^+-Nutrient RVD

We compared the effect of the potent calmodulin antagonists N-(4-aminobutyl) 5-chloro-2-naphthalenesulfonamide (W-13) and N-(6-aminohexyl)-5-chloro-1-napthalene sulfonamide (W-7), using the chloride-deficient derivative (W-12) as a hydrophobic control on hypotonic RVD and RVD occurring after cell swelling caused by L-Ala (Fig. 6). Both W-13 (100 μM) and W-7 (25 μM) prevented RVD after hypotonic swelling (Fig. 6*A*). Villus cells were larger when W-13 was added to the medium

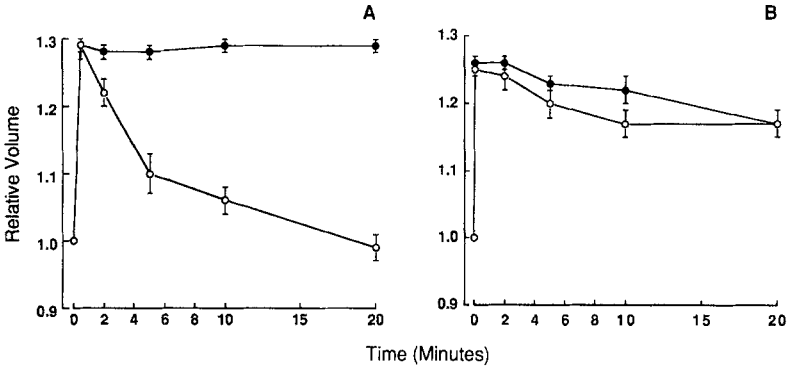
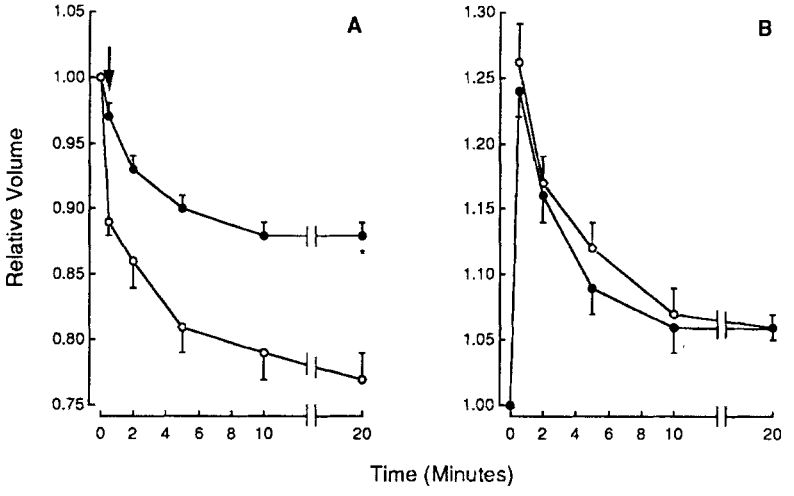


Fig. 4. Effect of A23187 on diltiazem or tetraethylammonium (TEA) inhibition of RVD. (*A*) ●, diltiazem (100 μM), 0.5 × isotonic; ○, A23187 (2.5 μM) in presence of diltiazem; $n = 5$. (*B*) ●, TEA (20 mM), 0.5 × isotonic; ○, A23187 + TEA; $n = 8$. Volume was measured electronically, expressed relative to isotonic control.



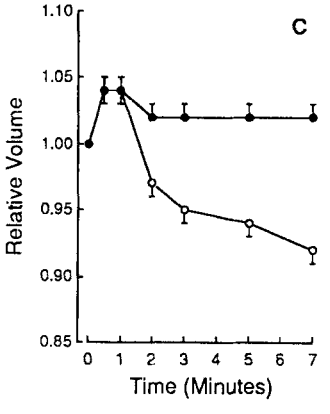


Fig. 5. Effect of charybdotoxin (CTX) on volume changes. (*A*) Calcium ionophore, A23187 (2.5 μM) added to villus cells in isotonic, Na^+-free ($NMDG^+$) medium with and without CTX (25 nM) ○, A23187; ●, CTX + A23187. (*B*) ○, 0.5 × isotonic, ●, 0.5 × isotonic with CBT (25 nM). Volume was measured electronically, expressed relative to isotonic control. (*C*) Effect of CTX on D-Glc RVD, ○, DGU, 20 mM; ●, CTX + 20 mM D-Glc; $n = 5$; $P < 0.005$.

compared with controls in W-12 (1.19 ± 0.01 *vs.* 1.06 ± 0.01, $P < 0.005$). There was more cell shrinkage in W-12 compared with W-13 (15.3 ± 2.3% *vs.* 0.3 ± 1.4%, $P < 0.005$). Conversely there was less shrinkage of cells in W-7 (25 μM) compared with controls in W-12 (6.0 ± 1.0% *vs.* 15.3 ± 2.3%, $P < 0.05$). In stark contrast, W-7 had no effect on RVD following cell swelling caused by L-Ala (Fig. 6*B*). There was no difference in the final relative volume of cells in the presence of W-7 compared with controls (0.94 ± 0.01 *vs* 0.91 ± 0.02, $n = 6$).

We determined the nature of calmodulin activation of K^+ and Cl^- conductances in villus entero-

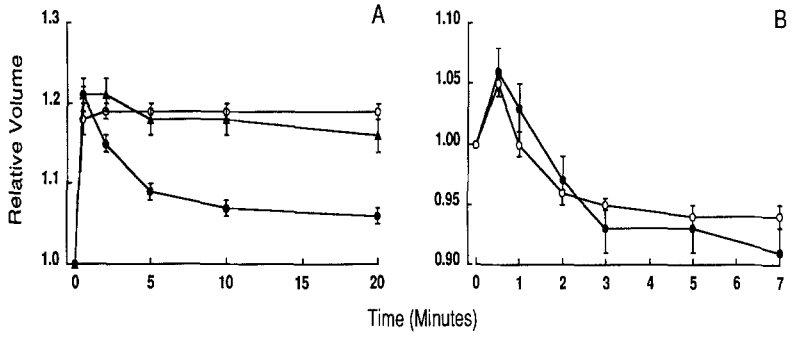


Fig. 6. Effect of calmodulin antagonists on RVD. (*A*) Hypotonic medium: ●, hydrophobic control W-12 (100 μM), 0.5 × isotonic; ○, W-13 (100 μM); ▲, W-7 (25 μM). (*B*) Volume changes after addition of 25 mM L-Alanine: ●, control; ○, W-7 (25 μM). $n = 5$ experiments performed in duplicate.

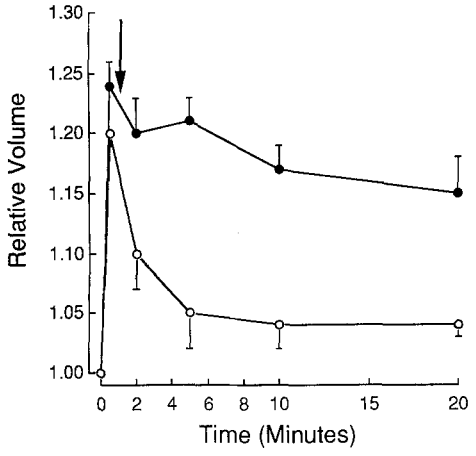


Fig. 7. Calmodulin antagonist W-13 (100 μM) bypassed by gramicidin (0.5 μM). ●, 0.5 × isotonic with W-13; ○, gramicidin added (arrow) at 35 sec after 0.5 × isotonic with W-13; $n = 7$. Volume was measured electronically, expressed relative to control.

cytes by using the cation ionophore gramicidin to bypass the W-13 inhibition of RVD. The rationale of this experimental approach, validated in lymphocytes (Grinstein et al., 1984), Ehrlich ascites cells (Hoffmann, Lambert & Simonsen, 1986) and villus enterocytes (MacLeod & Hamilton, 1991*a*) is that drugs that inhibit Cl^- conductance or both K^+ and Cl^- conductances cannot be bypassed with gramicidin (0.5 μM) since RVD will occur if and only if both K^+ and Cl^- efflux proceed. As illustrated in Fig. 7, addition of gramicidin allowed RVD to proceed in villus enterocytes hypotonically swollen in medium containing W-13. The final relative volume of cells to which gramicidin was added in medium containing W-13 was less compared with control cells in W-13 (1.04 ± 0.01 *vs.* 1.15 ± 0.03, $P < 0.005$).

Discussion

The volume decrease (RVD) of jejunal villus enterocytes swollen after the addition of L-Alanine, was unaffected in Ca^{2+}-free medium containing EGTA, while the RVD following hypotonic swelling of the same epithelial cells was prevented. Our demonstration of a need for extracellular Ca^{2+} to signal RVD in hypotonically swollen villus enterocytes is consistent with observations made in other epithelia (Wong & Chase, 1986; McCarty & O'Neil, 1990). The observed lack of extracellular Ca^{2+} dependency of L-Ala induced RVD raises the possibility that contamination of small amounts of extracellular Ca^{2+} might have been sufficient to elicit RVD in these cells; this explanation for our findings is unlikely since greatly increasing the cytoplasmic buffering power using the Ca^{2+} chelator BAPTA prevented this RVD. Our Ca^{2+} buffering experiments suggest that both models of RVD, hypotonic cell swelling and Na^+-nutrient cell swelling require increases in $[Ca^{2+}]_i$ to signal RVD. In the Na^+-nutrient model, the normal volume responses in Ca^{2+}-free medium suggest that RVD in this situation requires mobilization of intracellular Ca^{2+}, unlike the situation in hypotonic RVD, where the Ca^{2+} requirement is extracellular.

Both Ca^{2+} channel blockers used, nifedipine and diltiazem (Hosey & Lazdunski, 1988), prevented the hypotonic RVD but had no effect on the RVD following villus cell swelling caused by L-Ala addition. In other studies evaluating the impact of Ca^{2+} channel blockers, RVD of rabbit proximal straight tubule following hypotonic swelling was prevented by nifedipine and nitrendipine (McCarty & O'Neil, 1990) while in *Necturus* gallbladder, methoxyverapamil had no impact on hypotonic RVD (Foskett & Spring, 1985). Binding studies of enriched basolateral membranes of rabbit ileal villus enterocytes have demonstrated two types of Ca^{2+} channels; one restricted to interaction with phenylalkylamines, the other which interacted with dihydropyridines and benzothiazepines (Homaidan et al., 1989). The dihydropyridine blocker nifedipine is selective for inhibiting voltage-dependent Ca^{2+} channels of the L type (Hosey & Lazdunski, 1988), and previously it has been shown that cysteine-rich peptides cause a volume reduction when added to guinea pig villus enterocytes suspended in isotonic medium which was

prevented by either nifedipine (5 μM) or ω-conotoxin (MacLeod et al., 1991), therefore guinea pig villus enterocytes do appear to possess dihydropyridine-sensitive Ca^{2+} channels. In our experiments, the volume changes and the responses to calcium buffering observed are consistent with the interpretation that hypotonic RVD of jejunal villus cells requires a volume-induced Ca^{2+} influx pathway which is dihydropyridine sensitive.

The RVD of villus enterocytes hypotonically swollen by only 5 or 10%, to mimic the volume changes initiated by L-Alanine, was prevented by nifedipine. The epithelium of *Necturus* choroid plexus contains a cation-selective Ca^{2+} channel which hyposmotic stress opens (Christensen, 1987). These findings, from patch-clamp experiments, suggest that osmotic stress mechanically stretches the membrane activating a Ca^{2+} channel and permitting Ca^{2+} influx (Grinstein, DuPre & Rothstein, 1982). We speculated that the difference in the dihydropyridine sensitivity of RVD in hypotonic and Na^+-nutrient cell swelling might be due to the amount of osmotic stress generated by each type of challenge so that if Na^+-nutrient stimulated cell swelling were constrained to 5 or 10% of initial cell volume but the volume-induced Ca^{2+} pathway, which is dihydropyridine-sensitive, required $\geq 0.9 \times$ isotonic stress to be activated, then small amounts of hypotonic swelling would be dihydropyridine insensitive. However, this sensitivity was not observed. Our findings of the dihydropyridine sensitivity of hypotonic RVD are insufficient to label this volume-induced Ca^{2+} pathway either stretch activated or voltage dependent. They provide additional evidence that the hypotonic model of RVD differs in its Ca^{2+} signalling from the Na^+-nutrient model of RVD in jejunal villus cells.

Diltiazem but not TEA inhibition of RVD was bypassed with A23187 in hypotonically swollen enterocytes. We conclude that this inhibition occurred as a result of blocking Ca^{2+} influx through a volume-activated, dihydropyridine-sensitive Ca^{2+} pathway. Further evidence in support of this conclusion was found in the refractory nature of Na^+-alanine stimulated RVD to diltiazem. Previously, we demonstrated that K^+ channel inhibition prevented L-Ala induced swelling in villus enterocytes (MacLeod & Hamilton, 1991*a*). If diltiazem were inhibiting K^+ conductance, L-Ala cell swelling should have been prevented, but it was not. These data support the concept that hypotonic RVD requires extracellular Ca^{2+} and a volume-activated, dihydropyridine-sensitive Ca^{2+} pathway, while the RVD following cell swelling caused by Na^+-nutrient absorption does not utilize Ca^{2+} via a volume-activated, dihydropyridine-sensitive pathway.

Charybdotoxin (CTX) prevented RVD after cell swelling caused by D-glucose but had no effect on RVD after hypotonic swelling. CTX, while originally thought to have been a potent and selective blocker of Ca^{2+}-activated K^+ channels in several cell types (Miller et al. 1985; Giminez-Gallego et al., 1988; Grinstein & Smith 1989), has recently been shown to block both Shaker and mammalian Shaker-type K^+ channels (MacKinnon & Miller, 1989; Rehm, 1991). Lymphoid cells have been shown to possess a Ca^{2+}-activated CTX-sensitive K^+ channel, as well as a Ca^{2+}-independent but similarly CTX-sensitive K^+ channel (Grinstein & Smith, 1990; Grissmer, Lewis & Cahalan, 1992). The isotonic volume reduction in villus cells caused by the Ca^{2+} ionophore was prevented by a concentration of CTX previously demonstrated to inhibit Ca^{2+}-activated K^+ channels and prevent Ca^{2+} ionophore-induced hyperpolarization in lymphocytes (Grinstein & Smith, 1989). Our finding that CTX had no effect on hypotonic RVD in jejunal enterocytes suggests that in this model of RVD the K conductance activated is CTX insensitive. In contrast, the sensitivity of the Na^+-nutrient volume changes to CTX suggest that the K^+ conductance activated is CTX sensitive. These findings further support the interpretation that the Ca^{2+} signalling of ion conductances in response to osmotically active solute uptake is different compared with the response of swelling caused by passive hypotonic dilution.

Our data on the presence of RVD after hypotonic swelling to the calmodulin antagonists W-7 and W-13 are consistent with previous studies in epithelia and symmetrical cells and with data from the response to other calmodulin antagonists. Trifluoperazine, which prevents hypotonic RVD in *Necturus* gallbladder (Foskett & Spring, 1985), prevents hypotonic RVD and Ca^{2+} ionophore-induced volume changes in human lymphocytes (Grinstein et al., 1982). Hypotonically swollen *Amphiuma* red blood cells, which manifest RVD with a Ca^{2+}-activated K^+/H^+ exchange, were inhibited by chlorpromazine and trifluoperazine (Cala, 1983). Moreover, during hypotonic RVD and Ca^{2+} ionophore-mediated volume reduction in Ehrlich ascites cells, pimozide was found to inhibit both K^+ and Cl^- volume activated conductances. To circumvent any nonspecific effects on membrane-mediated events that these phenothiazine calmodulin blockers cause, we used the chlorine-deficient naphthalene sulfonamide derivative (W-12) as a hydrophobic control for W-7 and W-13 (Tanaka, Ohmura & Hidaka, 1982). That the calmodulin antagonist prevented hypotonic RVD, which could be bypassed with gramicidin, indicates that calmodulin regulates volume-activated K^+ conductance, but not the Cl^- conduc-

tance. The absence of an effect of the calmodulin antagonist on RVD following cell swelling caused by L-Ala addition is consistent with calmodulin neither activating nor regulating the K^+ and Cl^- conductances required for RVD after Na^+-nutrient cotransport.

The mechanisms by which ion transport pathways are activated for volume regulation are not well understood (Hoffmann & Simonsen, 1989; Grinstein & Smith, 1990; Pierce & Politis, 1990). While both passive, hypotonic swelling and cell swelling stimulated by Na^+-nutrient absorption elicit RVD by activating K^+ and Cl^- conductive pathways (MacLeod & Hamilton, 1991*a,b*), our studies of the Ca^{2+} mediation of these two models of RVD suggests that mechanisms of ion transport activation are very different in the two models. We do not know if our findings apply to studies of intact small intestine, but, in villus enterocytes isolated and studied in suspension, we speculate that ion conductances may be activated in hypotonically swollen villus enterocytes as follows. Hypotonic swelling activates Ca^{2+} influx via dihydropyridine-sensitive Ca^{2+} channels. Increased intracellular Ca^{2+} may alone promote 5-lipoxygenase activity resulting in increased leukotriene synthesis which activates Cl^- conductance. Leukotriene metabolites have been shown to accelerate RVD in hypotonically swollen Ehrlich ascites cells (Lambert, 1987) as well as activate Cl^- secretion in epithelia (Schaeffer & Zadunaisky, 1986). Increased Ca^{2+} together with calmodulin activate, via a phosphorylation event, the volume-sensitive K^+ conductance. This model is predicated on two features demonstrated in our current studies: the strict reliance on extracellular Ca^{2+} for RVD and the calmodulin dependence of volume-sensitive K^+ conductance. In contrast to the hypotonic model for activation of RVD, the insensitivity of the volume changes to extracellular Ca^{2+} and Ca^{2+} channel blockers, the charybdotoxin sensitivity of the D-Glu elicited RVD and the strict reliance of Na^+-nutrient volume changes as well as L-Ala stimulated ^{36}Cl efflux on protein kinase C (PKC) activity, all suggest a distinct model for RVD when cell swelling follows Na^+-nutrient absorption. Previously we have reported that the PKC inhibitors staurosporine and H-7 prevented L-Ala elicited RVD and ^{36}Cl efflux, but had no impact on hypotonic volume changes and ^{36}Cl efflux (MacLeod et al., 1992). Since PIP_2 hydrolysis will both increase $[Ca^{2+}]_i$ and diacylglycerol mass (Berridge, 1987) we speculate that Na^+-nutrient cotransport is coupled to PIP_2 hydrolysis; increased $[Ca^{2+}]_i$ could activate charybdotoxin-sensitive Ca^{2+}-activated K conductance, while diacylglycerol could activate the Cl^- conductance, with the net response being K^+ and Cl^- efflux, through conductive pathways and subsequent volume regulation.

In summary, jejunal villus enterocytes require extracellular Ca^{2+} for hypotonic RVD. Ca^{2+} channel blockers and intracellular Ca^{2+} buffering prevented RVD; charybdotoxin has no effect on hypotonic RVD, but a calmodulin antagonist prevented volume changes which occurred when the cation ionophore gramicidin was added. The cell swelling that occurred after Na^+ nutrient addition and the subsequent RVD were not altered by a Ca^{2+}-free medium or Ca^{2+} channel blockers, but this RVD was prevented by increased intracellular Ca^{2+} buffering. Calmodulin antagonists had no effect on Na^+-nutrient RVD, but volume regulation after cell swelling by D-gluc was prevented by charybdotoxin. Our results are consistent with the hypothesis that the Ca^{2+} mediation of ion conductances responsible for RVD after passive hypotonic cell swelling is different from cell swelling occurring because of transport of osmotically active Na^+-nutrient in these cells.

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