SIGNAL TRANSDUCTION OF VOLUME REGULATION IN VILLUS EPITHELIAL CELLS

by

R. John MacLeod

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Department of Medicine

Division of Experimental Medicine

McGill University, Montreal, Canada

Abstract

Volume increases of any magnitude in Na^+-absorbing jejunal epithelial cells result in the activation of K^+ and Cl^- channels; the resultant K^+ and Cl^- efflux causes a Regulatory Volume Decrease (RVD) restoring cell volume to normal. The objective of this thesis was to determine how the extent of volume increase was a determinant of the signaling of the K^+ channels responsible for RVD in guinea pig jejunal villus cells. We determined whether changes in intracellular pH (pH_i) were related to RVD after modest 5 to 7% volume increases, which duplicated the extent of cell swelling during Na^+-solute absorption, compared with larger volume increases observed after "standard" hypotonic (0.7 to 0.5 x isotonic) dilutions. Cell volume determinations were made using electronic cell sizing and pH_i was measured by fluorescent spectroscopy of villus cells in suspension. Modest 5 to 7% volume increases caused by slight hypotonic dilution caused an alkalinization of pH_i which was blocked by N-5-methyl-5-isobutyl-amiloride, (MIA) suggesting that Na^+/H^+ exchange (NHE) had been activated. RVD after the modest 5 to 7% volume increase was prevented by MIA or in Na^+-free medium. The order of potency of inhibition of RVD strongly suggested that NHE-1 was the isoform of NHE responsible for this alkalinization. The MIA-sensitive rate of pH_i recovery from an acid load was fastest in 5% hypotonic medium and slowest in 30% hypotonic medium suggesting activation of NHE was not a consequence of acidification. Greater volume increases caused by "standard" dilutions caused pH_i to acidify, but this acidification was increased by MIA, consistent with NHE inhibition. In cells swollen 7% in medium containing MIA to prevent RVD, NH_4Cl addition caused a

comparable alkalinization and consequent RVD suggesting that the alkalinization of pH_i was a requirement of osmolyte loss. Cells loaded with G protein antagonists did not show RVD after modest volume increase while cells loaded with G protein agonists exhibited MIA sensitive ΔpH_i suggesting a G protein mediated activation of NHE. Charybdotoxin (CTX) a maxi-K^+ channel inhibitor, blocked RVD after modest volume increase but had no effect on volume regulation after larger volume increases unless the pH_i was experimentally alkalinized. RVD after 15% volume increases was accelerated by phosphoprotein phosphatase inhibitors and blocked by a kinase inhibitor. Our studies suggest that different signal transduction pathways exist for activating K^+ channels required for RVD, but these pathways rely on the change in pH_i which accompanies cell swelling. It is the change in pH_i which is determined by the extent of the volume increase.

Résumé

Des augmentations plus ou moins importantes du volume des cellules épithéliales du jéjunum, actives dans l'absorption du Na^+, entraînent l'activation des canaux K^+ et Cl^-, le flux des ions K^+ et Cl^- qui en résulte amène une diminution régulatrice de volume (DRV) qui rétablit le volume normal de la cellule. L'objectif de la présente thèse est de montrer à quel point l'importance de l'augmentation de volume est un facteur déterminant de l'activation des canaux K^+, responsables de la DRV des cellules des villosités jéjunales chez le cochon d'inde. Nous avons déterminé s'il existait un lien entre les variations du pH intracellulaire (pH_i) et la DRV après de faibles augmentations de volume (5 à 7 %), qui reproduisaient le gonflement cellulaire durant l'absorption du Na^+ en solution, comparativement à des augmentations plus importantes de volume observées après des dilutions hypotoniques standard (dilutions de la solution isotonique de 0.7 à 0.5 fois). Les volumes cellulaires ont été déterminés par mesure électronique et le pH_i des cellules de villosité en suspension a été measuré par cytofluorométrie. De faibles augmentations de volume (5 à 7 %) causées par une légère dilution hypotonique ont entraîné une alcalinisation du milieu intracellulaire. L'alcalinisation peut être empêchée par l'addition de N-5-méthyl-5-isobutyl-amiloride (MIA), ce qui suggère que l'échangeur Na^+/H^+ (ENH) avait été activé par l'augmentation du volume. Après une faible augmentation de volume cellulaire (5 à 7 %), la DRV a été freinée par l'addition de MIA ou par un milieu exempt de Na^+. Le degré d'inhibition de la DRV suggère fortement que le ENH-1 est l'isoforme du ENH, responsable de l'alcalinisation du milieu cellulaire. Sensible à la présence de MIA, la vitesse de rétablissement de pH_i, suite à une charge acide, était la plus rapide dans un milieu

légèrement hypotonique (5 %) et la plus lente dans un milieu plus hyhpotonique (30 %), ce qui laisse croire que l'activation du ENH nétait pas une conséquence de l'acidification du milieu intracellulaire. De plus importantes augmentations de volume résultant de dilutions standard ont fait augmenter l'acidité. Par contre, cette acidification a été amplifiée par l'addition de MIA et l'inhibition du ENH qui en résulte. Pour des cellules gonflées (7 % de plus que le volume normal) dans un milieu contenant du MIA pour prévenir la DRV, l'addition de NH_4Cl a entraîné une alcalinisation comparable et la DRV prévue, suggérant que l'alcalinisation du milieu intracellulaire était un facteur nécessaire à la perte d'électrolytes. Les cellules ayant une forte concentration d'inhibiteurs de protéine G n'ont pas subi de DRV après une faible augmentation de leur volume, alors que les cellules ayant une forte concentration d'activateurs de protéine G montraient des $_{\Delta}pH_i$ dues au MIA, ce qui laisse croire que la protéine G serait un médiateur de l'activation du ENH. La charybdotoxine (CTX), un puissant inhibiteur du canal K^+, a freiné la DRV après une faible augmentation de volume, mais n'a eu aucun effet sur la régulation du volume après de plus importantes augmentations de volume, sauf dans les cas où le pH_i avait été diminué de façon expérimentale. La DRV après une augmentation de 15 % du volume était accélérée par les inhibiteurs de la phosphatase phosphoprotéique et freinée par un inhibiteur de la kinase. Notre étude suggère que différents mécanismes de transduction de signaux existent pour activer les canaux K^+, nécessaires à la DRV, par contre, ces mécanismes requièrent les variations de pH_i qui accompagnent le gonflement des cellules. L'importance de l'augmentation du volume détermine ces variations de pH_i.

Preface to the Thesis

This thesis is written in the form of original papers. The provision from the Thesis Guidelines states: "The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text (see below), of an original paper, or papers. In those cases the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction, and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion."

Four original manuscripts are presented, all of which have been submitted for publication, and in one case, extensively revised and resubmitted. Chapters Two to Five contain the manuscripts and include abstracts/summaries, introductions, materials and methods, results and discussion. Connecting texts have been inserted to provide continuity. In accordance with McGill University's thesis guidelines, this thesis includes a general abstract, an introduction and literature review, and a general discussion.

The experimental work described in Chapters Two to Five are in press or have been submitted for publications as follows:

Chapter Two: MacLeod, R.J. and J.R. Hamilton (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 September, 1996.

Chapter Three: MacLeod, R.J. and J.R. Hamilton (1996). G protein mediated activation of Na^+/H^+ exchange required for RVD after modest "physiological" volume increases in jejunal villus enterocytes. Am. J. Physiol. (Cell Physiol.)

Chapter Four: MacLeod, R.J. and J.R. Hamilton (1996). Increases in intracellular pH are required for K^+ channel activation after modest "physiological" swelling in villus epithelial cells. J. of Membrane Biology

Chapter Five: MacLeod, R.J. and J.R. Hamilton (1996). Signal transduction of K^+ channel activation for Regulatory Volume Decrease depends on extent of Epithelial Cell Swelling. J. of Membrane Biology

Peter Lembesis assisted in cell isolation and some volume determination experiments, while Carolyn Mandel typed the manuscripts.

The co-authors contribution to these papers was editorial. I initiated the experiments, designed and interpreted them, wrote the manuscripts and responded to reviewers' criticisms with new experiments and revisions.

All of the experiments in Chapter Two to Five began with one observation. While performing an experiment to measure changes in intracellular pH (pH_i) of

villus cells in response to Na^+-solute addition, to control for the extent of the volume increase ocurring because of Na^+-solute absorption, I hypotonically diluted these cells 5%. These "control" cells alkalinized, and the change in pH_i was prevented by an alkyl-amiloride derivative. All of the work in this thesis came from this unexpected observation. There is no place in the literature to celebrate a serendipitous finding, so all references to the fact that the activation of NHE in these cells was discovered while studying something else have been deleted from the manuscripts. Nevertheless, I will not forget the existential joy of finding something new and becoming indefatigable in the pursuit of its meaning.

Acknowledgements

I would like to thank Dick Hamilton, who gave me complete autonomy to initiate, interpret and write about experiments using crypt and villus epithelial cells. Without Dick's help over the last decade I would have learned little about grantsmanship. The most important lesson Dick taught me throughout this period is: Get your work published and put everything else in the rear-view mirror.

I would also like to thank Hugh Bennett (Dept. of Medicine, Royal Victoria Hospital) who convinced me to stay at McGill and always had an encouraging word for the only person in class with grey hair.

Finally, I wish to thank Jim Risk, Carolyn and Chris Pullen, Walley Schaber and C.P.S. "Ned" Franks, who I have repeatedly paddled with in the MacKenzie Mountains and the Barren Lands of the Northwest Territories. Each person, in different ways, taught me the mechanics of white-water canoeing. When I had been bludgeoned insensate by repeated dullness or the blandishments of consumerism, the only thing that ensured my return to the lab was a canoe trip. More importantly, these people have made me understand Canada as a renewing, dynamic and unexplored frontier, which is still rich in potential. This landscape of the imagination fuels the drive of my curiosity.

Dedication

The work in this thesis is dedicated to the memory of Hinda Kopelman, M.D., FRCP(C) (1953-1994) who I watched grow into a self-confident, productive scientist. I am sustained by recollections of Hinda at her best, listening wide-eyed to new findings and her infectious enthusiasm during collaborative projects.

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Chapter One

INTRODUCTION

The epithelial cells which line the mammalian jejunum play a fundamental role in the body's fluid and electrolyte homeostasis. The Na^+-absorbing cells found on the villus compartment of the jejunum which absorb Na^+ and Cl^- are the site of the transport of Na^+-coupled solutes such as D-glucose or L-alanine (Schultz, 1982; Schultz and Hudson, 1991). An example of the importance of villus cells to fluid and electrolyte homeostasis is found in diarrheal illnesses. Cholera is the most extensively studied severe diarrhea disease (Watten et al., 1959; Field et al., 1972; Field, 1977; Field and Semrad, 1993). Death from cholera is due to toxin induced secretion of fluid from the intestine with resultant hypovolemia and decreased circulation; it may be prevented by replacement of fluid intravenously or by oral-rehydration therapy. Cholera toxin inhibits Na^+ and Cl^- absorption in villus cells (MacLeod and Hamilton, 1987), but it has no effect on the capacity of these cells to absorb Na^+-coupled glucose. Consequently, glucose-based electrolyte oral-rehydration solutions have been proven to be extremely effective in the treatment of this diarrhea. Yet, it is seldom appreciated that absorption by villus cells of Na^+-coupled sugars or amino acids leads to an increased cellular content of osmotically active solutes which causes these cells to swell (MacLeod and Hamilton, 1991A). Because, in general, cellular volume maintenance occurs when net salt influx is equal to salt efflux (Hoffmann, 1978), the villus cells must lose electrolytes to regulate this transient volume change in order to decrease their volume back to normal. Clearly, increased insight into the regulation of electrolyte transport in

villus cells could provide an important basis for an improved understanding of nutrient, fluid and electrolyte homeostasis in health and in the management of diarrheal illnesses.

Jejunal villus cells can be studied in homogeneous suspensions and the mechanisms determining cellular responses to volume changes can be identified with precision. This thesis focuses on a key issue of volume regulatory processes during the course of Na^+-solute absorption in freshly isolated, suspended, mammalian jejunal villus cells.

I propose that it is the extent of initial swelling, which is the essential determinant of the villus cell's volume regulatory response. My findings are relevant to a consideration of intestinal Na^+-solute absorption where villus cell swelling is known to be modest in extent compared with that induced by more conventional osmotic challenges. They demonstrate that the volume to which a villus cell swells determines the signal transduction of the K^+ channels responsible for volume regulation. I propose that the activation of these K^+ channels relies on a change in intracellular pH (pH_i) that results from cell swelling and that this change in pH_i is strictly regulated by the extent of cell swelling.

Since cell volume is maintained by the equilibration of salt fluxes, the measurement of volume changes in homogeneous populations of suspended jejunal villus cells in response to volume increases of different amounts can provide

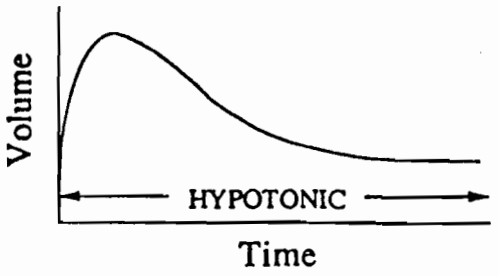
detailed insights into the mechanisms of regulation of ion channels and ion exchangers. Furthermore, new knowledge of the signal transduction pathways involved in epithelial cell volume regulation will help to elucidate the complexities of epithelial transport during secretion. Not only will this information advance our understanding of epithelial cell biology, it has the potential to generate new treatment concepts for the diarrheal diseases which continue to kill millions of the world's children each year (Snyder and Merson, 1982).

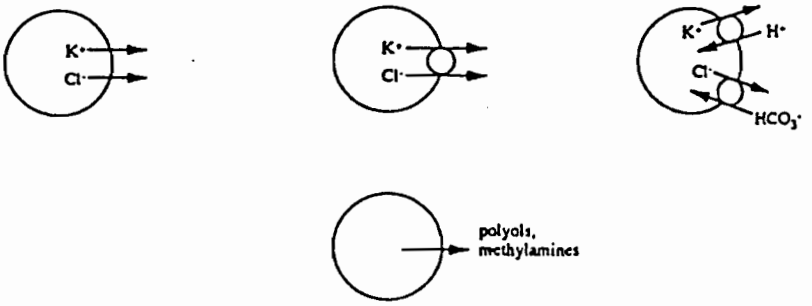
1. Principles of Volume Regulation

When animal cells in suspension are acutely exposed to an isotonic media, they initially behave as osmometers, increasing their volume as dictated by the water permeability of their membranes. When still suspended in the hypotonic medium, cells then undergo a slower, compensatory shrinkage because of the loss of osmolytes together with osmotically obliged water until their volume returns to normal (Figure 1A). This phenomenon is termed Regulatory Volume Decrease (RVD). Conversely, after first shrinking in a hypertonic or hyperosmotic medium, many cell types return to their initial volume, by a process termed Regulatory Volume Increase or RVI (Figure 1B). The effector mechanisms for these responses have been extensively investigated and are the subjects of recent reviews (Hoffmann and Dunham, 1995; Strange, 1994; Hoffmann et al., 1993. These

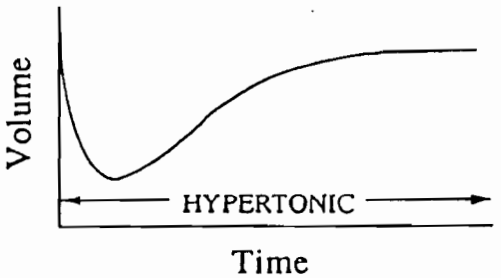
Figure 1: Illustrations of Regulatory Volume Decrease (RVD) and Regulatory Volume Increase (RVI) and different classes of transport systems shown to be involved for each in different cell types. Modified from Strange (1994).

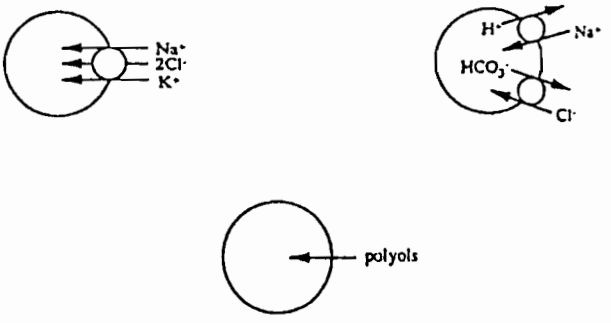
Ⓐ Regulatory Volume Decrease (RVD)





Ⓑ Regulatory Volume Increase (RVI)





mechanisms are intimately related to transepithelial ion transport, which in the intestine determines the movement of salts and water in and out of the body and hence diarrhea.

Because the inorganic ions K^+, Cl^- and HCO_3 comprise the bulk of osmotically active solute, it is not unreasonable that RVD in many cell types occurs because of a net loss of K^+ and Cl^-. This osmolyte loss which leads to a volume reduction may be mediated by a variety of ion channels and transporters (Figure 1A). For example, red blood cells from Amphiuma after swelling in hypotonic medium activate an electroneutral K^+/H^+ exchanger which is functionally coupled to Cl^-/HCO_3^- antiport. Because the net effect is the loss of KCl with water, the cells shrink. (Cala 1980). In avian and human red cells RVD occurs via KCl cotransport (Knauf, 1986; Hoffmann and Simonsen, 1989). In many other cell types KCl loss for RVD is via separate K^+ and Cl^- channels. Cells known to utilize this mechanism are lymphocytes (Grinstein et al., 1984B), Ehrlich ascites tumor cells (Hoffmann et al., 1986) and jejunal villus epithelial cells (MacLeod and Hamilton, 1991B).

In general, the RVI response is characterized by the uptake of Na^+ and Cl^-. (Figure 1B). There are two different mechanisms activated for this response, one being bumetanide-sensitive $NaKCl_2$ cotransport. Cells known to utilize this mechanism are Ehrlich ascites tumor cells (Levinson, 1990), avian red cells (Haas et al., 1982) and jejunal villus epithelial cells (MacLeod and Hamilton, 1990). The second mechanism is the activation of amiloride-sensitive Na^+/H^+ exchange (NHE)

which is functionally coupled to Cl^-/HCO^-_3 antiport. Examples are Amphiuma red cells (Cala, 1983), Necturus gallbladder cells (Ericson and Spring, 1982) and lymphocytes (Grinstein et al., 1985A). To date, all volume modes of activation of NHE require cell shrinkage. The coupling of NHE to Cl^-/HCO_3 is both indirect, by increasing $[HCO_3]$ that is a consequence of the increase in pH_i because NHE has been activated, or direct, due to deprotonation of regulatory pH-sensitive site on the antiporter (Grinstein and Foskett, 1990; Olsnes et al., 1987; Mason et al., 1989).

Kidney medullary cells are exposed to high osmolarities during antidiuresis, where the osmolality in this region of the mammalian kidney can reach levels higher than 1200 mOsM/kg. H_2O (Bankir and de Ronffignac, 1985). These cells regulate volume increase by upregulating Na^+ and Cl^- dependent transporters for betaine and myo-inositol resulting in cellular accumulation of these osmolytes (Kwon, et al. 1991).

This literature review will briefly discuss some mechanisms of osmotic activation of NHE and $NaKCl_2$ cotransport for volume increases, the role of Ca^{2+} in activating K^+ and Cl^- channels for volume decreases, and selected aspects of transcriptional regulation of organic osmolytes. It will then focus on how the activation of K^+ and Cl^- channels differ when villus epithelial cells swell because of Na^+-solute uptake compared with villus cell swelling because of hypotonic dilution.

1a. Osmotic activation of Na^+/H^+ exchange (NHE-1)

The magnitude and direction of net Na^+/H^+ exchange is determined by the difference in the transmembrane chemical potential differences for Na^+ and H^+, so that thermodynamically, net transport should cease when $[Na^+]_i/[Na^+]_o = [H^+]_i/[H^+]_o$. Depending on the cell type, this usually calculates to a pH_i of 7.8 - 8.0; but in most symmetrical cells examined NHE is virtually quiescent at normal isotonic volume and pH_i (Grinstein & Rothstein, 1986 D). The primary determinant of the rate of Na^+/H^+ exchange is pH_i. Earlier studies using kidney microvillus membrane vesicles clearly demonstrated rates of amiloride-sensitive ^{22}Na uptake, over a pH range of 6.62 to 7.47 which were not first-order (Aronson, et al., 1982) suggesting the existence of an allosteric modifier site on the Na^+/H^+ exchanger. When protonated, the modifier site was thought to activate the exchanger, which would protect the cytosol from excessive acidification, but as pH_i approached neutrality, deprotonation of the allosteric site would diminish activity of the exchanger (Aronson, 1985).

When the pH_i sensitivity of osmotically activated Na^+/H^+ exchange was recognized, two important concepts emerged. The first was that cytoplasmic acidification did not precede osmotic activation of Na^+/H^+ exchange. The second concept was revealed from kinetic analysis of Na^+/H^+ exchange activity as a function of pH_i in hypertonic compared with iso-osmolar medium. The exchanger is

nearly quiescent at normal pH_i in iso-osmolar medium. This pH_i is referred to as the "set point" of the exchanger. However, in the hypertonic medium at normal pH_i large amounts of activity were observed, and the pH_i where activity was barely detectable was shifted ~ 0.4 pH units in the alkaline direction (Grinstein et al., 1984; Grinstein and Rothstein, 1984; Grinstein et al., 1985A). These studies and others (Grinstein and Rothstein, 1986D) suggested osmotic stimulation of the exchanger was a consequence of changes in properties of the exchanger but not alterations in the driving forces or concentrations of allosteric activators.

This kinetic modification of the Na^+/H^+ exchanger's activity, an upward displacement in the pH_i "set point" of the modifier site was also observed in a variety of cells treated with mitogens or hormones (reviewed in Grinstein et al., 1989; Wakabayashi, et al., 1992). Because there was no additive response of cells treated with mitogens and hypertonicity experiments were interpreted to suggest osmotic activation and growth factors were activating Na^+/H^+ exchange through a common mechanism (Grinstein, et al., 1986C; Bianchini, L. et al., 1991). In addition, both osmotic activation and mitogenic activation of Na^+/H^+ exchange required intracellular ATP since metabolic depletion of ATP prevented both types of activation. Together, these findings of the same kinetic effects on the Na^+/H^+ exchanger stimulated by receptor mediated mitogens or cell shrinkage in hypertonic medium, the lack of an additive effect between these two stimuli and the absolute requirement for ATP for both stimuli suggested phosphorylation of the exchanger was responsible for activation.

1b. Is protein kinase C responsible for osmotic activation of NHE-1?

Tumor promoters like phorbol esters will activate Na^+/H^+ exchange in many cell types and this activation is kinetically manifested as an upward displacement in the pH_i "set point" of the modifier site. There was no additive effect on Na^+/H^+ exchange activation after phorbol ester treatment and hypertonicity suggesting that protein kinase C mediated the osmotic activation of the exchanger (Grinstein et al., 1985). However, in hypertonically shrunken cells no decrease in phosphatidylinositol 4,5 bisphosphate levels were observed, nor were increases in IP_3 or migration of protein kinase C from cytosol to membrane detected (Grinstein et al., 1986 A). Importantly, in cells depleted of protein kinase C by down-regulation with high doses of phorbol esters, while Na^+/H^+ exchange was no longer responsive to tumor promoter, when treated hypertonically the Na^+/H^+ exchanger was activated (Grinstein et al., 1986B). Therefore, these studies suggested that the osmotic activation of Na^+/H^+ exchange was not mediated by diacylglycerol increases subsequent to protein kinase C activation.

1c. Cloning and structure-function studies of Na^+/H^+ exchange (NHE-1)

Further understanding of the mechanism of osmotic activation of Na^+/H^+ exchange was promoted by studies which identified the gene of human fibroblast Na^+/H^+ exchange (Sardet et al., 1989). Termed NHE-1, this Na^+/H^+ exchanger is an integral membrane glycoprotein of ≈ 105kDa. Hydropathy plots of the deduced

amino acids suggest that NHE-1 has two domains: a hydrophobic N-terminal region which has ten putative transmembrane segments and a hydrophilic C-terminal region thought to extend into the cytosol. Of the predicted 815 amino acids, ≈ 500 comprised the N-terminal and membrane spanning regions and ≈ 300 were in the C-terminal region (Sardet, et al., 1990). Using deletion mutants of NHE-1, it was subsequently established that the N-terminal transmembrane domain was sufficient both for insertion of the exchanger and ion transport since removal of the entire cytoplasmic domain preserved 25% of the exchanger's activity. However, deletion of the cytoplasmic domain shifted the "set point" of the exchanger in the acidic direction. The implication of this observation was that the H^+ modifier site was located within the N-terminal transmembrane domain but that the C-terminal region of NHE-1 controlled the "set point" value of the exchanger (Wakabayashi et al., 1992). Use of a specific antibody against a β- galactosidase — Na^+/H^+ antiporter fusion protein revealed that NHE-1 increased phosphorylation in response to mitogens (Sardet et al., 1990). However, in a subsequent study, when all putative phosphorylation sites were removed in a deletion mutant, this maneuver had no effect on growth-factor induced phosphorylation of the exchanger. Phosphopeptide maps of wild type and deletion mutant exchangers revealed that all phosphorylation sites, including growth-factor sensitive ones, mapped to the cytoplasmic tail, yet, deletion of all of these sites reduced by only 50%, growth factor induced alkalinization. Importantly, individual replacement of serine residues on the tail with alanine had no effect on growth-factor activation of exchange activity. These data clearly suggested a mechanism not requiring direct phosphorylation of NHE-1 in response to mitogens. It was speculated that an ancillary regulator protein,

These data clearly suggested a mechanism not requiring direct phosphorylation of NHE-1 in

response to mitogens. It was speculated that an ancillary regulator protein, perhaps a kinase substrate, might exist, and that the regulatory protein controlled the set point value of the exchanger (Wakabayashi et al., 1994).

1d. Is NHE-1 activated by phosphorylation for Regulatory Volume Increase?

No, NHE-1 is not activated by phosphorylation for RVI. The definitive experiments which support this conclusion used a polyclonal antibody raised against the carboxy terminus of the human NHE-1 isoform of the antiporter to immunoprecipitate NHE-1 of ^{32}P - labelled human bladder carcinoma and CHO cells (Grinstein et al., 1992). Constitutive phosphorylation of NHE-1 was observed under isotonic conditions. Hypertonic activation of these cells caused Na^+ dependent, N-5-methyl-5-isobutyl-amiloride sensitive alkalinization of pH_i; but while phosphorylation of NHE-1 was also observed, the degree of phosphorylation was no different from that observed in isotonic medium. Further experiments assessed whether the pH_i increases after hypotonicity were due to an isoform of NHE which was not detected by the antibody used in the study. Using fibroblasts that were deficient in functional NHE-1 it was first established that these cells did not respond to hypertonic challenge with an alkalinization of pH_i and immunoprecipitation experiments did not detect the 110 kDa phosphoprotein. When these cells were transfected with cDNA for human NHE-1, the transfectants possessed under isotonic conditions a phosphorylated, immunoreactive

"That was the curious incident", remarked Sherlock Holmes.

Like the dog in the Holmes story (Doyle, 1913) the requirement of ATP for hypertonic activation of NHE-1, when it is clear that the antiporter is not directly phosphorylated, provides some insight into the mechanism of osmotic activation. Previous studies had shown inhibition of basal NHE activity by metabolic depletion of ATP in lymphocytes (Grinstein et al., 1985), A431 carcinoma cells and smooth muscle cells (reviewed in Grinstein et al., 1994). Depletion of ATP prior to hypertonic shrinkage also inhibits volume regulation and NHE activity (Bianchini et al., 1991). A subsequent study revealed that inhibition of NHE-1, induced by ATP depletion, was a function of pH_i; inhibition was greatest near "physiological" pH_i but partially overcome by intense cytosolic acidification. However, once again, there was no difference in phosphorylation of immunoprecipitates of NHE-1 from isotonic untreated cells compared with ATP depleted cells; suggesting inhibition of NHE-1 exerted by metabolic depletion did not require dephosphorylation of NHE-1 (Gross et al., 1994).

The two themes of the ATP requirement for both isotonic and osmotically activated NHE-1 and a putative involvement of the cytoskeletion in NHE-1 activation began to converge when immunological experiments indicated that NHE-1 was observed in regions of focal accumulation on the surface of fibroblasts or CHO cells where vinculin, tailin and actin were also observed (Grinstein et al., 1993). This sequestration of NHE-1 was thought to reflect an interaction with cytoskeletal elements

first hydrophobic stretch and the carboxy-terminal cytosolic domain are poorly conserved. NHE-1 has been localized to the basolateral membrane of both villus and crypt epithelial cells of mammalian small intestine (Tse et al., 1991; Bookstein et al., 1994A), while NHE-3 has been localized to the brush border membrane of villus cells and rabbit proximal tubule cells (Bookstein et al., 1994A; Biemesderfer et al., 1993). NHE-2 has been reported to be localized to the brush border membrane of ileal villus cells (Hoogerwerf et al., 1994).

The isoforms vary in their sensitivity to amiloride and amiloride analogues. NHE-3 is highly resistant to inhibition by amiloride or its N-5-alkyl derivatives (Orlowski, 1993; Tse et al., 1993). NHE-1 and NHE-2 are equal sensitive to amiloride and its derivatives but clonidine is more potent than cimetidine inhibiting NHE-2 and -3 while this order is reversed for NHE-1 (Yu et al., 1993). NHE-2 is not sensitive to extracellular K^+ while NHE-1 is competitively inhibited by high K^+ (Yu et al., 1993; Orlowski, 1993). Studies using transfected antiporter deficient fibroblasts have shown that NHE-1 and NHE-2, are both stimulated by hypertonicity and inhibited by hypotonicity (Kapus et al., 1994). In contrast, the apical isoform, NHE-3 is inhibited by hypertonic cell shrinkage but unaffected by the same degree (0.5 X isotonic) of hypotonicity. Clearly, chimeric constructs of NHE-1 and NHE-3 will implicate those portions of the antiporter that are responsible for this tonic responsiveness. NHE-4 has been transfected into antiporter deficient fibroblasts. Immunoblot analysis demonstrated cross-reactivity to a polyclonal antibody made to a NHE-4 fusion protein. In contrast with the other three isoforms, NHE-4 was quiescent in acid-loaded

in rat stomach and a subset of tubules in the collecting duct of the inner medulla of rat kidney (Bookstein et al., 1994B). These data suggest sufficient kinetic and pharmacological data exist from studies of transfectants of these NHE isoforms to distinguish volume and pH_i responses in epithelial cells having both apical and basolateral isoforms of NHE.

3. Osmotic activation of $NaKCl_2$ cotransport by phosphorylation

As described above, and illustrated in Figure 1, many cell types which exercise RVI after hyperosmotic shrinkage do so by activating an electroneutral $NaKCl_2$ cotransporter. Because this loop diuretic sensitive cotransporter is the source of Cl^- influx for continued Cl^- secretion from secretory epithelia (Greger et al., 1988) considerable research effort has sought mechanisms for its activation. Stimulation by secretogogues like cAMP cause secretory epithelia to undergo an isotonic volume reduction because K^+ and Cl^- efflux is greater than Na^+ and Cl^- influx (Foskett, 1994; Macleod et al., 1994A). With continued Cl^- secretion stimulated by cAMP, $NaKCl_2$ is activated; consequently it was considered a likely kinase substrate. Hypertonic shrinkage of shark rectal gland resulted in increased phosphorylation of immunoprecitated $NaKCl_2$ cotransport protein ($\approx$125kDα) at both serine and threonine residues. This phosphorylation was temporally correlated with increased ^3H-benzametanide binding suggesting hyperosmotic activation of $NaKCl_2$ cotransport occurred via phosphorylation. The activity of $NaKCl_2$ cotransport in avian erythrocytes is increased by hypertonicity, and increased further with okadaic acid, an inhibitor of

phosphoprotein phosphatases 1 and 2A, findings suggesting the cotransporter has been activated by a kinase (Pewitt et al., 1990; Palfrey, 1994).

The best evidence that hypertonic shrinkage activates this cotransporter with direct phosphorylation would be to show that after site-directed mutagenesis, hypotonicity does not affect activity of the cotransporter. With the recent cloning and functional expression of a bumetanide- sensitive $NaKCl_2$ cotransporter from Squalus, such structure-activity experiments are presumably in progress (Xu et al., 1994). Therefore, while phosphorylation does not directly activate NHE-1, there is compelling evidence to suggest that osmotic activation of $NaKCl_2$ cotransport is via direct phosphorylation. Nevertheless, nothing is understood about how a putative kinase is activated with the change in cell volume.

4. Changes in intracellular calcium, and volume-activated K^+ and Cl^- channels

As described above, many cell types, after swelling regulate their volume (Regulatory Volume Decrease, RVD) by the efflux of KCl, through separate K^+ and Cl^- conductive pathways or channels. The calcium dependency of this RVD has been the subject of numerous recent reviews (Foskett, 1994; Hoffmann et al., 1993; Pierce and Politis, 1990). In general, a generic model has emerged which requires cell swelling after hypotonic dilution to activate Ca^{2+} permeable channels. Intracellular Ca^{2+} then increases; in some cases it is liberated from an intracellular source. Subsequent to the increase of $[Ca^{2+}]_i$, a high-conductance Ca^{2+} activated K^+ channel is activated, together

5. High conductance Ca^{2+} - activated K^+ ($maxi\text{-}K^+$) channels and RVD

Because Ca^{2+} was demonstrated a generation ago to activate K^+ channels in red blood cells (Gardos, 1958), earlier studies of volume regulation focused on Ca^{2+} as the sole mediator of swelling-induced K^+ channel activation (Grinstein et al., 1982). Extensive reports showing inhibition of RVD in both symmetrical and epithelial cells by "selective" drugs and toxins (i.e. quinine and apamin) were interpreted to suggest Ca^{2+} activated K^+ channels were responsible for osmolyte loss (reviewed in Hoffmann et al., 1993; Hoffmann and Simonsen, 1989). These high conductance Ca^{2+} activated K^+ ($maxi\text{-}K^+$) channels have single-channel conductances >100pS (Latorre et al., 1989). Charybdotoxin (CTX), a peptide isolated from the venom of Lieurus quinquestriatus blocks these channels with high affinity in several epithelia (Garcia, et al., 1995; Cornejo et al., 1984; Lu et al., 1993) while other $maxi\text{-}K^+$ channels are CTX-insensitive (Reinhart et al., 1991). Three mechanisms can modulate $maxi\text{-}K^+$ channel activity: pH_i, $[Ca^{2+}]_i$ and protein phosphorylation. Acidification of pH_i will inhibit $maxi\text{-}K^+$ channel activity in Necturus choroid plexus (Christensen & Zenthen, 1987) and cultured renal medullary thick limb cells (Cornejo et al., 1990). Alkalinization of pH_i (6.9 to 7.9) will increase $maxi\text{-}K^+$ channel activity in Necturus gallbladder when $[Ca^{2+}]_i$ is elevated (Copello et al., 1991). These mechanisms of regulation are further discussed in Chapter Four, but the literature available to date on $maxi\text{-}K^+$ channels and RVD has focused on the relationship between $[Ca^{2+}]_i$ increases and activation of this family of channels.

of changes in intracellular Ca^{2+} in mediating the K^+ and Cl^- channels required for volume decreases (RVD). As illustrated in Figure 2(A), the presumption which underlies these experiments, is that cell swelling activates some transporters, whereas cell shrinkage activates others. The activation of these ion channels or antiporters / cotransporters are independent of each other. An alternative view is that there is a regulator in cells consisting of a volume-sensing mechanism plus a signalling apparatus that communicates with both swelling — and shrinkage-induced transporters (Parker, 1993, 1994). The model illustrated in Figure 2(B) suggests activation of swelling — and shrinkage-induced transporters are in some way coordinated. The model system used to develop a model of coordinate regulation of volume are red blood cells from rabbit, human and dog, where KCl cotransport is activated by cell swelling and NHE is activated by cell shrinkage.

7a. "Macromolecular crowding": Kinetic argument

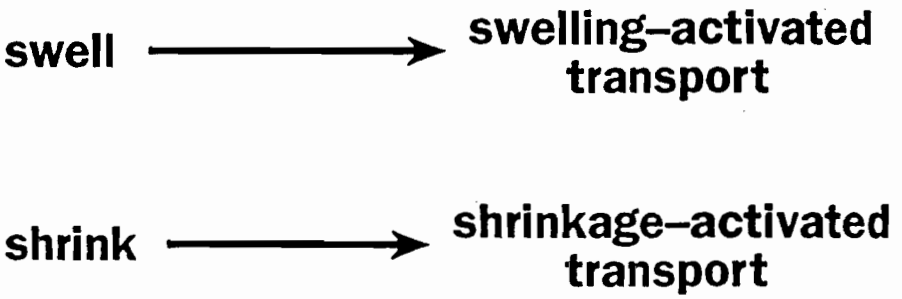
It was first shown using rabbit (Jennings et al., Rohil, 1990; Jennings and Schultz, 1991) and human (Kaji and Tsuketani, 1991) red cells that okadaic acid and calyculin A, inhibitors of phosphoprotein phosphatases, increase the lag between initial cell swelling and the activation of KCl cotransport. In contrast, the rate of deactivation of KCl cotransport observed after shrinking the cells back to their normal volume was not influenced by these inhibitors. A kinetic model of these results suggested that activation of KCl cotransport by red cell swelling involves a net dephosphorylation (either the cotransporter itself or some regulatory protein). In other words, the more

phosphorylated the KCl cotransporter (or a putative unidentified protein) is, the greater the inactivation of the system. Subsequent experiments using dog red cells showed that activating KCl cotransport and inactivating NHE with swelling took the same amount of time, but that triggering shrinkage-induced NHE and inactivating KCl contransport was much faster than the swelling-stimulated events (Parker et al., 1991). This recurrent pattern of shrinkage and swelling induction suggested some sort of coordinate regulation. It is of interest that the first explicit proposal of such coordinate regulation, essentially cycles of dephosphorylation and phosphorylation as well as latter commentators (Coussins, 1991; Palfrey, 1994), presumed that KCl cotransport or NHE were directly dephosphorylated or phosphorylated, respectively. This presumption conveniently overlooked an important set of experiments.

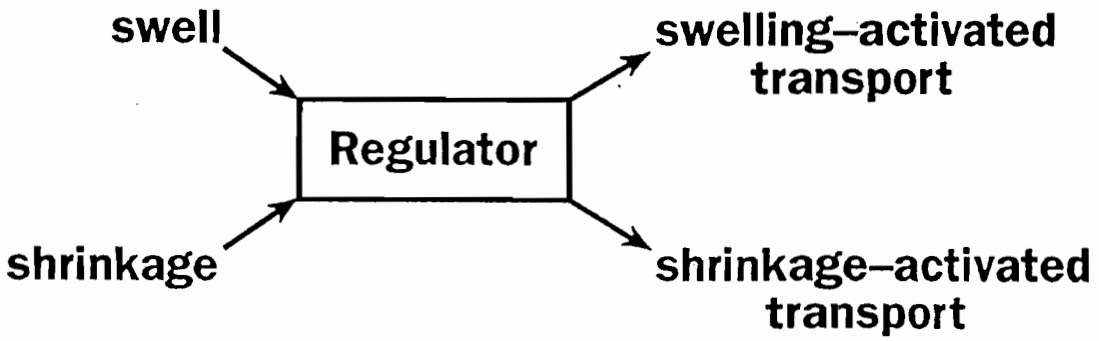
7b. "Macromolecular crowding": Experimental Evidence

Using dog red cells and red cell ghosts, studies suggested the signal for activation of shrinkage-induced NHE and swelling-induced KCl cotransport originated with attainment of a critical cytosolic protein concentration rather than a specific cell volume (Colclasure and Parker, 1992). KCl cotransport was activated in ghosts at a volume that was ~ 70% less than the activation volume for intact cells,

Figure 2: Schematic representation of the linkage, or lack of linkage between swelling-induced and shrinkage-induced transporters. (A) shows the two transporters responding to cell swelling or shrinkage independently; (B) indicates the responses are in some way coordinated. Modified from Strange (1994).


swell
swelling–activated transport
shrink
shrinkage–activated transport

A


swell
Regulator
shrinkage
swelling–activated transport
shrinkage–activated transport

B

but at a similar haemoglobin concentration as the intact cells. Furthermore KCl cotransport in re-sealed ghosts at a fixed protein concentration became quiescent at a protein concentration similar to that at which shrinkage-induced NHE became activated. Their results suggest that the intracellular protein concentration in the re-sealed ghosts defined both the activation of NHE and the inactivation of KCl cotransport, and implied the two transporters were under control of a common regulator. Parker speculated that swelling and shrinking are translated into a chemical signal by the influence of macromolecular crowding on the relevant kinases and phosphatases. The principal concept of "macromolecular crowding" is that a change in the activity coefficient of macromolecules will be produced by changes in the mole fraction of the solvent. The space occupancy by large solute particles can exert large effects (one to three orders of magnitude) on the activity coefficients of other large solute molecules. Activation or inactivation of transporters would result from changes in the reactivity of a soluble component eg. a kinase or phosphatase (Minton et al., 1992; Garner and Burg, 1994). Consequently, relatively small and presumably nonspecific changes in cytosolic concentrations of macromolecules could have profound effects on the activity of different transporters. While the experimental data supporting a role for "macromolecular" crowding are elegant, other studies using red cell ghost preparations suggested swelling activation of KCl contransport is not mediated by dilution of any cytoplasmic solute (Sachs and Martin, 1993). Further progress in assessing whether macromolecular crowding has a role in volume regulation awaits identification of the molecular species responsible for KCl cotransport. The concept

of coordinate responses may explain some aspects of volume regulation in red blood cells, but the findings in this dissertation refute most predictions that "macromolecular crowding" would make regarding the signalling of volume regulation in absorptive epithelial cells. See below, and MacLeod (1994).

8. Regulation of organic osmolyte transport; hypertonicity-responsive element in BGTI gene

As illustrated in Figure One, the last mechanism of volume regulation to be discussed is the loss of organic osmolytes like β-taurine for RVD (reviewed in Hoffmann et al., 1993) or the synthesis of organic osmolytes like myo-inositol, glycine betaine or sorbitol which result in RVI. The major difference between the accumulation of such solutes compared with the activation of NHE or $NaKCl_2$ cotransport is that while the latter is activated immediately after cell shrinkage, synthesis of the former takes from 24 hours to several days (Garcia-Perez and Burg, 1991). These non-perturbing "compatible" organic osmolytes glycine betaine and β-taurine are accumulated secondary to an increase in Na^+ and Cl^- uptake. Assays of RNA abundance and nuclear run-on assays revealed increasing tonicity increased the rate of transcription for the sodium-chloride-betaine (BGTI) transporters 24 hours after hypertonic challenge (reviewed in Handler and Kwon, 1993). The 5' flanking region of the BGTI gene was studied using standard transfection analysis of different constructs with luciferase reporter genes in response to hypertonicity. Electrophoretic shift assays allowed precise

identification of a tonicity-sensitive element (Takenaka et al., 1994). This approach will undoubtly resolve the exact molecular details of how extracellular hypertonicity activates transcription of osmotically sensitive genes like aldose reductase and BGTI.

9. Osmoprotective accumulation of glycerol in yeast: MAP kinase

Like eukaryotes described above, yeast cells in their natural environment are exposed to increases and decreases in osmolarity. The relative ease by which yeast may be exploited genetically, together with some recent findings merit inclusion in this review because the resolution provided by this approach may point the way of future studies required to understand the molecular details of transporter and ion channel activation during volume regulation in animal cells.

Hypertonic solutions stimulate in Saccharomyces cerevisiae the synthesis of glycerol (reviewed in Ammerer '94). A mitogen-activated protein kinase (MAP kinase) termed HOG1 (High Osmolarity Glycerol) is tyrosine phosphorylated after hypertonic shock in yeast (Brewster et al., 1993) A homologue of HOG1, p.38 is also tyrosine phosphorylated in response to hypertonicity in lymphocytes and macrophages (Han et al., 1994). HOG1 appears to be activated by a two-component histidine / aspartate kinase similar to that used by bacteria to respond to hypertonicity (Maeda et al., 1994). Genetic complementation experiments allowed cloning of a gene, the hydropathy plot of which contained four hydrophobic

peptides that are presumably transmembrane. This protein — Sho1p — was postulated to be a transmembrane osmosensor. Because Sho1p contained a Src homology domain (SH3), it follows Sho1p would bind a target protein through sequences containing proline and hydrophobic amino acids (Schlessinger, 1994). The protein that Sho1p binds to was then shown to be the MAP kinase (Pbs2p) responsible for activating HOG1 (Maeda et al., 1995). While the identification of Sho1p as a membrane osmosensor is quite tantalizing, as yet it is unclear whether MAP kinase is involved in regulation of either osmolyte transporter genes or transporters themselves in higher eukaryotes.

In contrast with the HOG pathway, the PKC 1 kinase cascade is required for yeast growth at low osmolarity (Davenport et al., 1995). The major difference between these two pathways is that while HOG activation takes several hours, one minute after hypotonic shock an increase was observed in tyrosine phosphorylation of the first MAP kinase pathway in the PKC 1 kinase cascade. Furthermore, there was a strict correspondence between the extent of tyrosine phosphorylation and the magnitude of hypotonicity (Davenport et al., 1995). Clearly, genetic complementary experiments with the appropriate mutants will shed enormous insight into this kinase's activation by hypotonicity as well as the identity of substrates.

10. Summary of sections 3 to 8 (Fig. 2)

Illustrated in Fig. 2A, and discussed above, is the concept that the activation of ion channels responsible for RVD is independent of the activation of transporters responsible for volume increase (RVI) after cell shrinkage. Increases in $[Ca^{2+}]_i$ are required for complete RVD suggesting Ca^{2+} directly or indirectly via Ca^{2+} / calmodulin activates the K^+ Cl^- efflux responsible for volume reduction. There is some evidence that swelling and shrinkage-activated transport may be coordinately regulated, in some cells, by cycles of dephosphorylation and phosphorylation. This concept of a coordinate response is illustrated in Fig. 2B. In contrast with cells where volume regulation is rapid ($t_{0.5} \approx 15$ min), some animal cells and yeast which generate osmolytes like glycerol or glycine betaine to increase their volume, do so over several hours. This de novo synthesis of both osmolyte transporters has allowed detailed analysis of regulation of transcription. However, to reiterate, the mechanism by which a cell knows, or senses that it has become larger and how this signal is transduced to activate the transporter responsible for a consequent volume regulation is not known.

11. How a villus cell swells is a determinant of the signalling pathways that activate RVD

As mentioned above, the mechanism whereby Na^+-absorbing jejunal villus epithelial cells exercise RVD after swelling caused by suspension in hypotonic (0.5 or 0.7 X isotonic) media, is the activation of K^+ and Cl^- channels (MacLeod and Hamilton, 1991B). The major "work" these cells perform is the Na^+-coupled uptake of glucose and amino acids. Cotransport of these substrates with Na^+ across the apical membrane also leads to a volume increase. The first demonstration that villus enterocytes manifested RVD after exposure to D-glucose or L-alanine used electronic cell sizing to measure volume changes in these cells when 20 to 25 mM of the Na^+-solute was added to the cells suspended in isotonic medium (MacLeod and Hamilton, 1991A). Peak volume increases of 5 to 7% of the enterocytes isotonic volume occurred 1 to 2 minutes after solute addition. Once maximal volume was reached, the cells began to shrink, completing RVD by 2 minutes (D-glucose) or 3 minutes (L-alanine). Cell swelling did not occur in the absence of extracellular Na^+, or after addition of the non-Na^+ cotransported isomer (D-alanine or L-glucose). Furthermore, cell swelling caused by alpha-CH_3-D-glucoside was prevented by phlorizin, a well characterized inhibitor of the SGLT1 isoform of the Na^+-glucose cotransporter. Together, these data were consistent with the volume increase of the enterocyte being due to the influx of Na^+ with either D-glucose or L-alanine, together with water.

Different classes of K^+ or Cl^- channel blockers prevented the RVD after villus cell swelling caused by Na^+-solute addition. Furthermore, when cells were maximally swollen after L-alanine addition but then permeabilized with the ionophore gramicidin, they continued to swell in Cl^- containing but not Cl^--free medium. These data suggested the RVD after cell swelling caused by Na^+-nutrient absorption was also due to the activation of K^+ and Cl^- channels.

11A. Effects of Protein kinase C (PKC) inhibitors on RVD

The first evidence that different signalling mechanisms were responsible for activation of the K^+ and Cl^- channels responsible for RVD in these two models of cell swelling came from volume and $^{36}C1$ efflux measurements using PKC inhibitors (MacLeod et al., 1992A). Both 1- (5-isoquinolinylsulfonyl 1) - 2 methyl piperizine (H-7) and the fungal alkaloid, staurosporine, prevented RVD after L-alanine induced cell swelling but had no effect on RVD of cells swollen by hypotonic dilution (either 50% or 5% to duplicate the extent of the L-alanine stimulated volume increase). Increases in the rate of ^{36}Cl efflux that were stimulated by hypotonic dilution were inhibited by a chloride channel blocker but were not affected by the PKC inhibitor, while L-alanine-stimulated increases in this rate were inhibited an equivalent amount by either the PKC inhibitor or the chloride channel blocker. Importantly, gramicidin permeabilization of these cells in isotonic medium stimulated volume changes that were not influenced by the PKC inhibitors, suggesting that Na^+ entry alone was not activating the PKC-sensitive Cl^- channel. These experiments

suggested that PKC was required to activate the Cl^- channel required for RVD after cell swelling stimulated by L-alanine. In contrast, the Cl^- channel activated by swelling caused by hypotonic dilution did not require PKC for activation.

11B. The Ca^{2+} requirement for RVD in hypotonic and Na^+ - nutrient RVD

The second line of evidence that the signalling mechanisms responsible for activating the K^+ and Cl- channels was dependent on the mode of the volume increase came from studies of the Ca^{2+} requirement for RVD (MacLeod et al., 1992 B). Enterocytes, hypotonically diluted so they swelled to a volume (5 - 7%) comparable to the one generated by L-alanine absorption, showed no RVD in Ca^{2+} - free medium. In contrast, volume changes stimulated by L-alanine were not affected in the same Ca^{2+} - free conditions. However, buffering putative changes in $[Ca^{2+}]_i$ by loading the cells with a calcium buffer, BAPTA, blocked RVD in both models of cell swelling. This finding was consistent with both modes of cell swelling resulting in an increase in $[Ca^{2+}]_i$, but the hypotonic model required Ca^{2+} influx from an extracellular source while $[Ca^{2+}]_i$ was mobilized for the Na^+-solute induced volume changes. Subsequent experiments suggested this Ca^{2+} was mobilized from an IP_3-sensitive pool (MacLeod, 1994). Consistent with this interpretation, both dihydropyridines and inorganic Ca^{2+} channel blockers (Ni^{2+}, Co^{2+}) prevented RVD after cell swelling following hypotonic dilution but had no effect on RVD after swelling caused by L-alanine absorption. Furthermore, the napthalene sulfonamide derivatives W-13 and W-7 prevented RVD after swelling caused by hypotonic

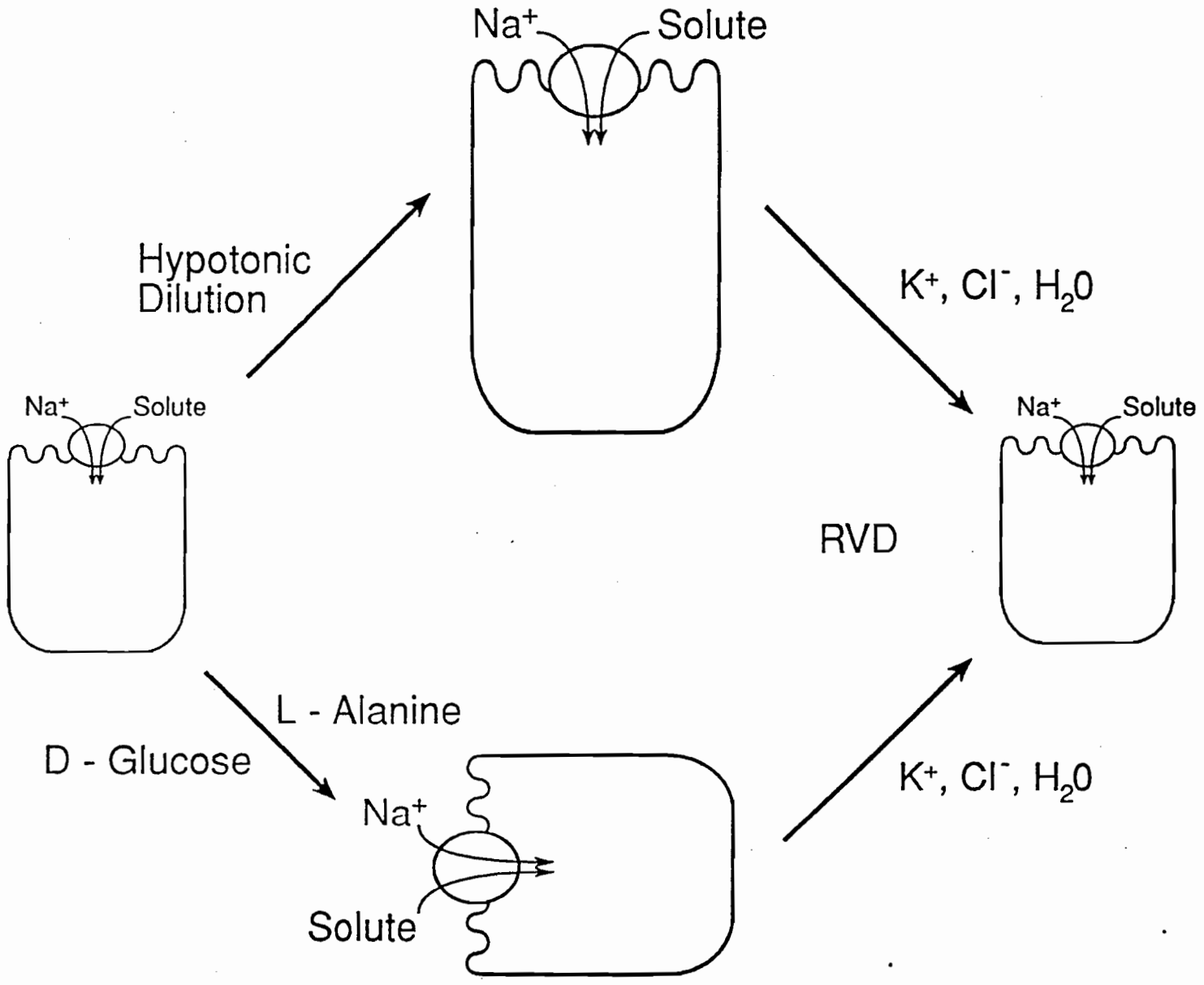
dilution, but had no effect on RVD caused by addition of L-alanine. The inhibition of RVD by these calmodulin antagonists could be bypassed by gramicidin, suggesting that the K^+ channel required calmodulin for activation, whereas the K^+ channel activated for RVD after Na^+-nutrient swelling was calmodulin-insensitive. Charybdotoxin (CTX), the potent and selective inhibitor of maxi-K^+ channels described above, blocked RVD after cell swelling induced by D-glucose but had no effect on RVD after cell swelling caused by hypotonic dilution.

12. Concluding comments

From this review, it can be concluded that signalling of the K^+ and Cl^- channels responsible for RVD is substantially different when cell swelling is initiated by hypotonic dilution compared with Na^+-solute absorption. Clearly, the mechanism of cell swelling is a determinant of ion channel activation required for RVD. These experiments also suggested a cotransporter "works" in intact cells to couple the activation of ion channels responsible for volume regulation with the volume increase caused by solute absorption (reviewed in MacLeod, 1994). Thus, it appears that in Na^+-absorbing jejunal villus epithelial cells, while K^+ and Cl^- channels are activated for RVD, it is the mechanism of cell swelling (resulting in the same osmolyte loss) which is responsible for the different signalling pathways (Figure 3).

Throughout hundreds of experiments measuring Na^+-nutrient stimulated villus cell swelling, I have been impressed by the fact that the maximal volume

Figure 3: Illustration of two ways to make villus enterocyte swell. Suspension in hypotonic medium causes a volume increase followed by RVD due to activation of K^+ and Cl^- channels (upper). Addition of either D-glucose or L-alanine causes a volume increase of 5 to 7% which is followed by RVD also due to activation of K^+ and Cl^- channels (lower). While the same osmolyte loss is responsible for RVD in both models of villus cell swelling, the mechanism of cell swelling is different and leads to different signal transduction of ion channel activation for the volume recovery. Modified from MacLeod and Hamilton (1991A,B) and MacLeod et al., (1992A,B).


Na+
Solute
Hypotonic
Dilution
K+, Cl-, H20
Na+
Solute
RVD
Na+
Solute
L - Alanine
D - Glucose
Na+
Solute
K+, Cl-, H20

increase was never more than 5 to 7% of the isotonic cell volume. This observation suggested that there was a "physiological" volume increase associated with Na^+ - solute cotransport. While the above cited work used hypotonic dilutions of 5 to 10% to duplicate this volume increase, clearly showing that the Cl^- channel did not require PKC for activation and that the Ca^{2+} source was extracellular, the mechanism of K^+ channel activation was not explored in detail. If there is a "physiological" volume increase, then the extent of villus cell swelling might be a determinant of the signalling of the ion channels responsible for RVD.

The work in this dissertation supports the thesis that the extent of villus cell swelling is a determinant of the signal transduction of the K^+ channels responsible for volume regulation. I propose that the activation of the K^+ channels required for RVD relies on the change in pH_i that results from cell swelling and that this change in pH_i is strictly dictated by the extent of cell swelling.

13. Specific Aims

The original research to be described in the body of this thesis was carried out to determine:

1. Is the response to cell swelling, RVD, determined by the extent of cell swelling?
2. What determines changes in pH_i during cell volume increases?

3. What activates the mechanism responsible for the change in pH_i during modest volume increases?
4. Is there a causal relationship between volume change induced shifts in pH_i and the activation of CTX-sensitive K^+ channels?
5. Is there a relationship between pH_i, phosphorylation, and the calmodulin-sensitive K^+ channel required for RVD after marked osmotic challenge?

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Chapter Two

Activation of Na^+/H^+ exchange is required for Regulatory Volume Decrease after modest `physiological' volume increases in jejunal Villus Epithelial Cells

Summary

Epithelial cell volume increases which occur because of the uptake of Na^+-cotransported solutes or hypotonic dilution are followed by a Regulatory Volume Decrease (RVD) due to the activation of K^+ and Cl^- channels. We studied the relationship of Na^+/H^+ exchange (NHE) to this RVD in suspended guinea pig jejunal villus cells, using electronic sizing to measure cell volume changes. When the volume increase achieved by these cells during Na^+-solute absorption is duplicated by a 5 or 7% hypotonic dilution, their intracellular pH (pH_i) (monitored by fluorescent spectroscopy in cells loaded with 2,7, bis(carboxyethyl)-5-(6)-carboxyfluorescein), alkalinizes, a response which is blocked by N-5-methyl-5-isobutyl-amiloride (MIA, 1 μM). RVD was also prevented by MIA or by Na^+-free medium. The order of potency of amiloride and non-amiloride derivatives inhibitors MIA>5-(N,N-dimethyl)amiloride >cimetidine>clonidine, strongly suggest that NHE-1 is the isoform of NHE responsible for the pH_i changes observed. When the MIA-sensitive rate of pH_i recovery from an acid load (dpH_i/dt) was determined in media of different tonicities, pH_i recovery was fastest in 5% hypotonic medium and slowest in 30% hypotonic medium, suggesting that activation of NHE-1 was not a consequence of cellular acidification. In contrast, substantial hypotonic dilution caused larger volume increases followed by RVD which was not affected by MIA or Na^+-free medium. A 30% hypotonic dilution caused the pH_i to acidify but this acidification was increased by MIA, suggesting that NHE inhibition occurs when volume increases are relatively large. In cells hypotonically diluted 7% in MIA to block

RVD, NH_4Cl caused alkalinization and consequent RVD whereas propionic acid caused acidification, but no volume response. We conclude that in Na^+ absorbing enterocytes, the NHE-1 isoform of Na^+/H^+ exchange is stimulated by volume increases which duplicate the `physiological' volume increase occurring when these cells absorb Na^+-cotransported solutes. The subsequent alkalinization of pH_i is a required determinant of the osmolyte loss which underlies this distinct volume regulatory mechanism.

Introduction

A modest, 5 to 7% volume increase of a jejunal villus epithelial cell is of interest because it duplicates the size to which these cells swell during absorption of Na^+-solute (1,2,3). With any such volume increase, these cells activate K^+ and Cl^- channels causing a Regulatory Volume Decrease (RVD); the resultant KCl efflux returns the volume to normal (4,5). Because reports using symmetrical mammalian cells have suggested that intracellular pH (pH_i) is a determinant of volume regulation (6,7,8), our efforts to characterize RVD after modest 5 to 7% swelling focused on pH_i and its relationship to Na^+/H^+ exchange (NHE) activity as cell volume was increased experimentally. In this report, using suspended jejunal villus epithelial cells, exposed to a slight hypotonic challenge (0.95 x isotonic) to duplicate the volume increase occurring because of Na^+-solute absorption (5 to 7% swelling), we show a precise relationship between NHE activity, pH_i and the activation of ion channels for RVD. This sequence of events differs greatly from that observed after a `standard' hypotonic challenge (0.70 to 0.50 x isotonic) where after cell swelling of 15 to 20%, NHE activity is inhibited (9,10,11). NHE has been identified in many cell types (12,13) and four distinct isoforms have been cloned (14-18). We have characterized NHE activation and its relationship to RVD by measuring changes in cell volume, pH_i, ^{22}Na influx and rates of pH_i recovery from an acid load. By showing distinct differences in the response to cell swelling of different magnitudes, we have provided new insight into the mechanism of signal transduction for volume regulation in absorptive epithelial cells.

Materials and Methods

Solutions and reagents

Volume measurements were made on cells suspended in Na^+ medium at a density of 30,000 cells mL^{-1}. This medium contained (in mM): 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 10 Hepes (pH 7.3, 295 mOsm). Na^+-free medium and K^+ rich medium were made by isosmotic replacement of NaCl with the chloride salts of N-methyl-D-glucamine ($NMDG^+$) and K^+, respectively, and titrated to pH 7.3 with the corresponding bases. Isotonic low Na^+ medium contained 25 mM NaCl with 115 mM $NMDG^+$. Salts were adjusted so that after 5% or 30% hypotonic dilution the final concentration of Na^+ was 25 mM. Na^+ uptake buffer was Na^+ medium supplemented with bovine serum albumin (type V) at 1 mg/mL.

We purchased bafilomycin A_1 from Dr. K. Altendorf (Universitat Osnabruck, Germany). The acetoxymethylester of 2', 7'-bis (carboxyethyl) 5- (6)-carboxyfluorescein (BCECF) was obtained from Molecular Probes (Eugene, OR). 5-N-methyl-N-isobutyl amiloride (MIA) and 5-(N,N-dimethyl) amiloride (DMA) were from Research Biochemicals (Natick, MA) and $NMDG^+$ from Aldrich (Milwaukee, WI). Nigericin, cimetidine and clonidine were from Sigma Chemical (St. Louis, MO), and RPM1-1640 (10x) medium from GIBCO/BRL (Burlington, Ont.). Di-nonylpthalate was from Pfaltz and Bauer (Waterbury, CT), and $^{22}NaCl$ was purchased from Amersham (Montreal, Quebec).

Villus cell isolation and volume determination

Villus cells were isolated from segments of adult male (200-300g) guinea pig jejunum by mechanical vibration as previously described (19). We resuspended

isolated cells at 0.8-1.5x10^6 cells/µl in RPM1-1640 medium (without HCO_3) containing bovine serum albumin (type V) at 1 mg/ml and 20m Na Hepes, pH 7.3 at 37°C. Viability, assessed by trypan blue exclusion was 85%, 3 hr after suspension in medium. Cell volume was measured using a Coulter Counter (model ZM) with an attached Channelyzer (C-256) as previously described (1,2,4). Villus cell volume measured electronically over a range of tonicities correlated (r=0.967) with direct measurements of cell water (4). The effect of amiloride and non-amiloride inhibitors illustrated in Fig. 8 was made using an attenuation setting of 32. We determined relative cell volume as a ratio of cell volume under study conditions to the volume under basal conditions in isotonic medium.

pH_i Measurement and Manipulation

For the fluorimetric determination of pH_i, villus cell suspensions (1 x 10^6 cells/mL in Hepes-RPM1) were loaded with BCECF by incubation with the parent acetoxymethylester (3.7 µM) for 15 min at 37°C. After washing, 0.5 to 0.8 x 10^6 cells were used for fluorescence determination in 2 ml of the indicated medium using a Hitachi F-4000 fluorimeter with excitation at 495 nm and emission at 525 nm using 5 and 10 nm slits, respectively. We acid loaded cells by preincubating 10^6 cells/ml for 5 min in RPM1 medium containing 2.5 mM NH_4Cl at 37°C, followed by sedimentation and resuspension in 2 ml of the indicated NH_4^+-free medium. For experiments using acid-loaded cells, loading with NH_4^+ and BCECF was performed simultaneously as described by others (20). The MIA sensitive rate of pH_i recovery was the difference between pH_i recovery in the presence of bafilomycin (100 nM) and Zn^{2+} (100 µM), and pH_i recovery in the presence of these inhibitors and 1µM

MIA. Rates of pH_i recovery were determined in low Na^+ medium (25 mM) as described above. Calibration was performed in K^+ medium with nigericin (21) using a quench correction factor as described (22).

Uptake of ^{22}Na

We measured the initial rate of ^{22}Na influx modifying a procedure previously described (19). Each villus cell preparation was divided in half and resuspended at a final concentration of 5-6 mg protein/mL in prewarmed uptake medium in a continuously stirring cuvette. This medium contained 10 μM bumetanide to inhibit $NaKCl_2$ cotransport. Uptake was initiated with the addition of ^{22}Na at a concentration of 8-10 μCi/mL. Immediately afterwards, a 500 μL cell suspension was removed and added to 500 μL of ice-cold, 0.1M $MgCl_2$. This aliquot which took <5s to obtain was taken to represent extracellular ^{22}Na associated with the cell pellet. Uptake was terminated after 90 s by diluting 500 μL of cell suspension in an equal volume of ice-cold, 0.1 M $MgCl_2$, which was then gently layered on a 100 μL layer of di-n-butylphalate: di-n-nonylphalate (3:2 vol) and centrifuged in an Eppendorf microfuge for 20 s. Aliquots of the supernatant were saved for counting and the cell pellet was processed as previously described (2,4,19). Prior to the addition of ^{22}Na, duplicate samples were taken and processed as above, but following aspiration of the supernatant and oil, 100 μL of Triton X-100 was added to the pellet. After vigorous shaking and cell lysis, we measured protein concentration using the Bio-Rad protein assay reagent (Bio-Rad, Richmond, CA) with bovine γ-globulin as a standard. All uptake experiments were done in the presence and absence of 1 μM MIA. For the uptake experiments performed under 5% hypotonic conditions, the medium was diluted with distilled H_20, and 60s later

the isotope was added and uptake was allowed to proceed for 90s. In preliminary experiments we determined that ^{22}Na uptake was first order for 110s. The extracellular ^{22}Na associated with the cell pellet was subtracted from the 90s values. Rates, expressed as nmoles/min/mg protein, were based on 5 to 7 experiments performed in duplicate.

Statistics

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

Results

Changes in pH_i with villus cell swelling

The resting pH_i of villus cells in Hepes-buffered RPM1-1640 medium (nominally HCO_3 free) was 7.39 ± 0.04, n=45. Figure 1 illustrates the changes in pH_i of villus cells in suspension hypotonically diluted 5% or 30%. To mimic the volume increase that occurs because of the uptake of either L-alanine or D-glucose (11,12), the villus cells were diluted 0.95 x isotonic (Fig 1A). This dilution, which generated a modest volume increase and caused the pH_i to acidify 0.03 ± 0.01 pH units (n=16), was followed by alkalinization. This alkalinization after 0.95 x isotonic dilution was prevented by 1 µM 5-(N-methyl-N-isobutyl) amiloride (MIA) (Fig. 1B). After a 0.70 x isotonic dilution which generated a substantial volume increase, the cells acidified 0.105 ± 0.041 pH units (n=16) (Fig. 1C). Unlike cells suspended in 0.95 x isotonic, these cells continued to acidify over 3 min and MIA (1µM) increased this acidification (Fig 1D). These pH_i changes are summarized in Fig. 2. A 0.95 x isotonic dilution caused ΔpH_i/3 min of 0.070 ± 0.010 pH units which was abolished by 1 µM MIA (0.020 ± 0.10 pH units; $p<0.005$). Similarly, a 0.93 x basal dilution caused MIA sensitive alkalinization (ΔpH_i: 0.050 ± 0.010 vs 0.01 ± 0.01, $p<0.001$, n=16). In contrast, 0.70 x isotonic dilution caused acidification which was increased by MIA (1µM) (ΔpH_i: -0.016 ± 0.005 vs -0.030 ± 0.005, $p<0.005$, n=16).

5-(N-methyl-N isobutyl) amiloride sensitive Regulatory Volume Decrease

Figure 3 illustrates the relationship between Regulatory Volume Decrease (RVD), the amiloride derivative MIA and extracellular Na^+. In regular Na^+ medium

Figure One: Effect of 0.95 x isotonic or 0.70 x isotonic dilution on pH_i of villus cells in suspension. (A) 0.95 x isotonic. (B) N-5-methyl-N-isobutyl amiloride (MIA, 1μM) +0.95 x isotonic. (C) 0.70 x isotonic. (D) MIA + 0.70 x isotonic. Tracings corrected for dilutional artifact, and are representative of 16 replicate experiments. Results of one experiment illustrated. An example of an uncorrected trace and a description of how the dilution artifact was corrected is found in the Annex of this thesis.

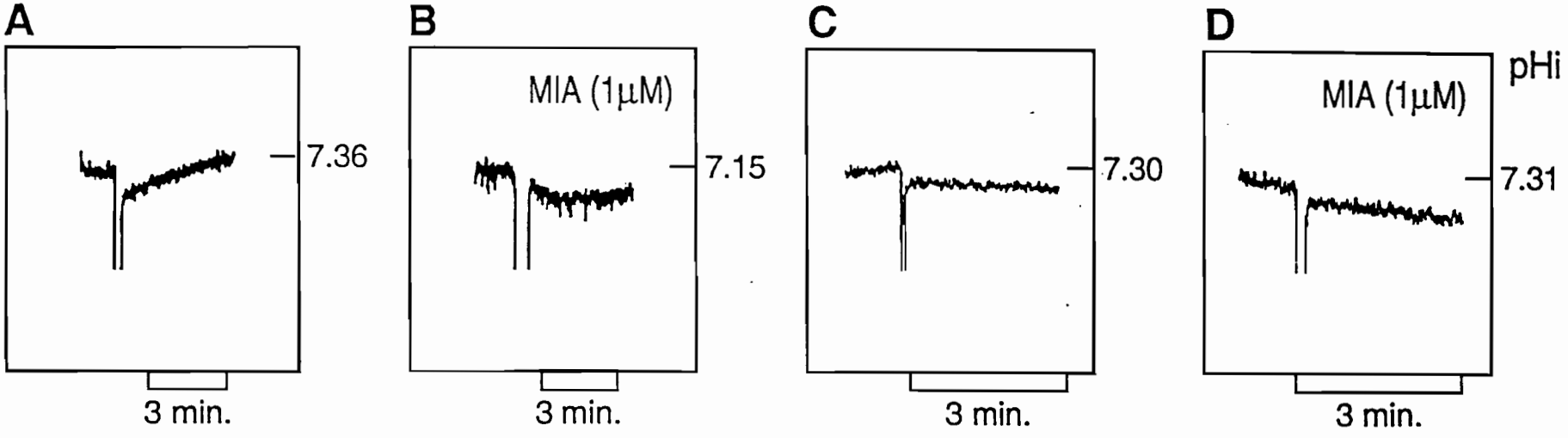
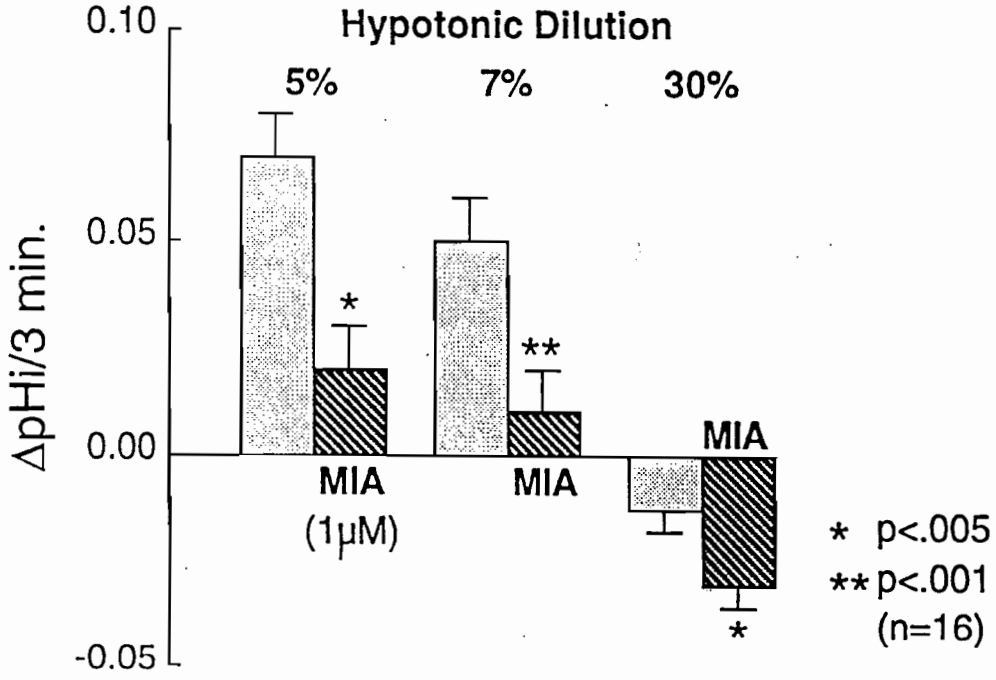

A
7.36
3 min.
B
MIA (1μM)
7.15
3 min.
C
7.30
3 min.
D
pHi
MIA (1μM)
7.31
3 min.

Figure Two: Summary of pH_i changes in the absence or presence of N-5-methyl-N-isobutyl amiloride (MIA) after 0.95 x isotonic, 0.93 x isotonic or 0.70 x isotonic dilutions. MIA (1μM), *$p<0.005$, **$p<0.001$, n=16.


Hypotonic Dilution
5%
7%
30%
0.10
0.05
0.00
-0.05
ΔpHi/3 min.
*
**
*
MIA
MIA
(1μM)
MIA
* p<.005
** p<.001
(n=16)

(140 mM Na^+) villus cells diluted 0.95 x isotonic rapidly swell, then exercise RVD, returning to their basal volume in ~ 4min (Fig 3A). This RVD was prevented by 1 μM MIA (final relative volume: 1.03 ± .01, $p<0.001$, n=6). When we replaced all medium Na^+ isotonically with N-methyl-D-glucamine, RVD following 0.95 x isotonic dilution was prevented (final rel. volume: 1.04 ± .01, $p<0.001$, n=6, Fig. 3B). The RVD after 0.93 x isotonic dilution was also prevented by 1 μM MIA (final relative volume: 1.05 ± .01, $p<0.001$, n=6; Fig 3C). RVD after 0.93 x isotonic dilution was also prevented in Na^+-free medium; final rel. volume: 1.05 ± .01 $p<.001$, N=6, Fig. 3D).

The effect of Na^+-free medium on the RVD following greater volume increases occurring in a very hypotonic medium is illustrated in Fig. 4. After 0.7 x isotonic dilution or a 0.8 x isotonic dilution the subsequent RVD was complete. Consistent with these findings, MIA (1 μM) had no effect on RVD of villus cells after 0.7 x isotonic dilution in Na^+ containing medium (extent of volume decrease: 19 ± 1% vs 19 ± 1%, n=6). Together the data illustrated in Figs 1 to 4 suggest that when the villus cell swells after a 5% hypotonic dilution, the pH_i undergoes a MIA sensitive alkalinization and complete volume recovery is both Na^+-dependent and MIA sensitive.

<u>^{22}Na influx increased by 0.95 x isotonic dilution</u>

We measured the initial rate of ^{22}Na influx of villus cells in suspension that was MIA sensitive under isotonic conditions or after 0.95 x isotonic dilution. Under isotonic conditions (140mM Na^+) measured in the presence of bumetanide (10μM)

Figure Three: Effect of 5-(N-methyl-N-isobutyl) amiloride (MIA) and Na^+-free medium on regulatory volume decrease (RVD) after 5% or 7% hypotonic dilution. (A) O, 0.95 x isotonic; ●, 0.95 x isotonic, MIA (1μM). (B) ●, 0.95 x isotonic in Na^+-free medium. (C) O, 0.97 x isotonic; ●, 0.97 x isotonic, MIA (1μM). (D) ●, 0.93 x isotonic in Na^+-free medium. $p<0.001$ in all cases where RVD was inhibited. n=6. Volume measured electronically, expressed relative to isotonic control.

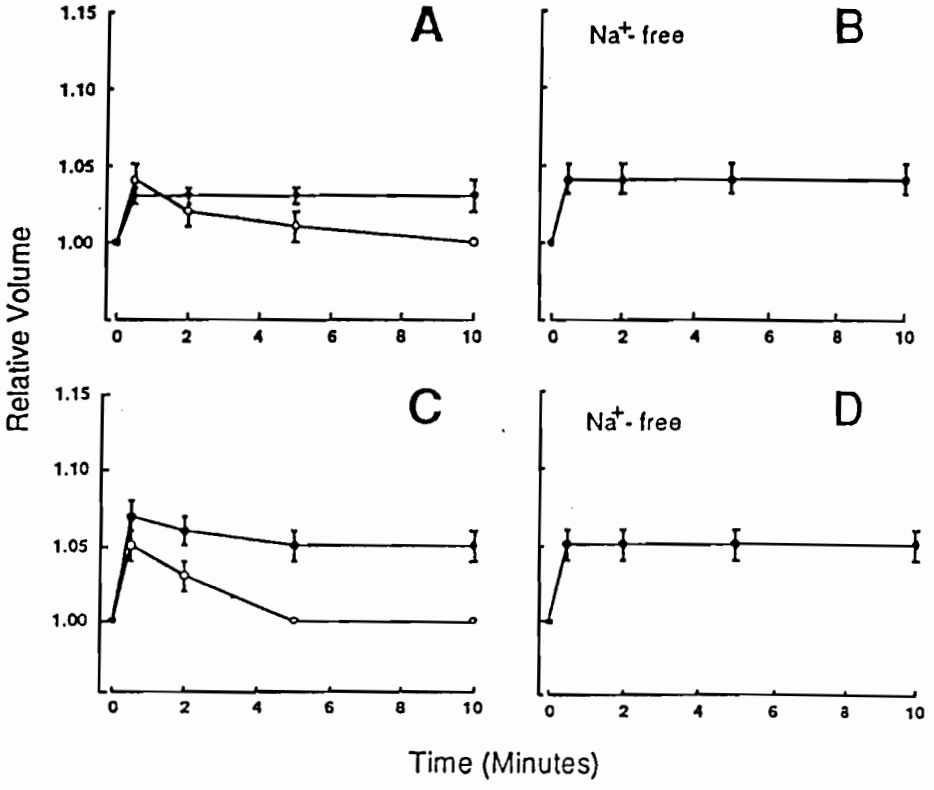
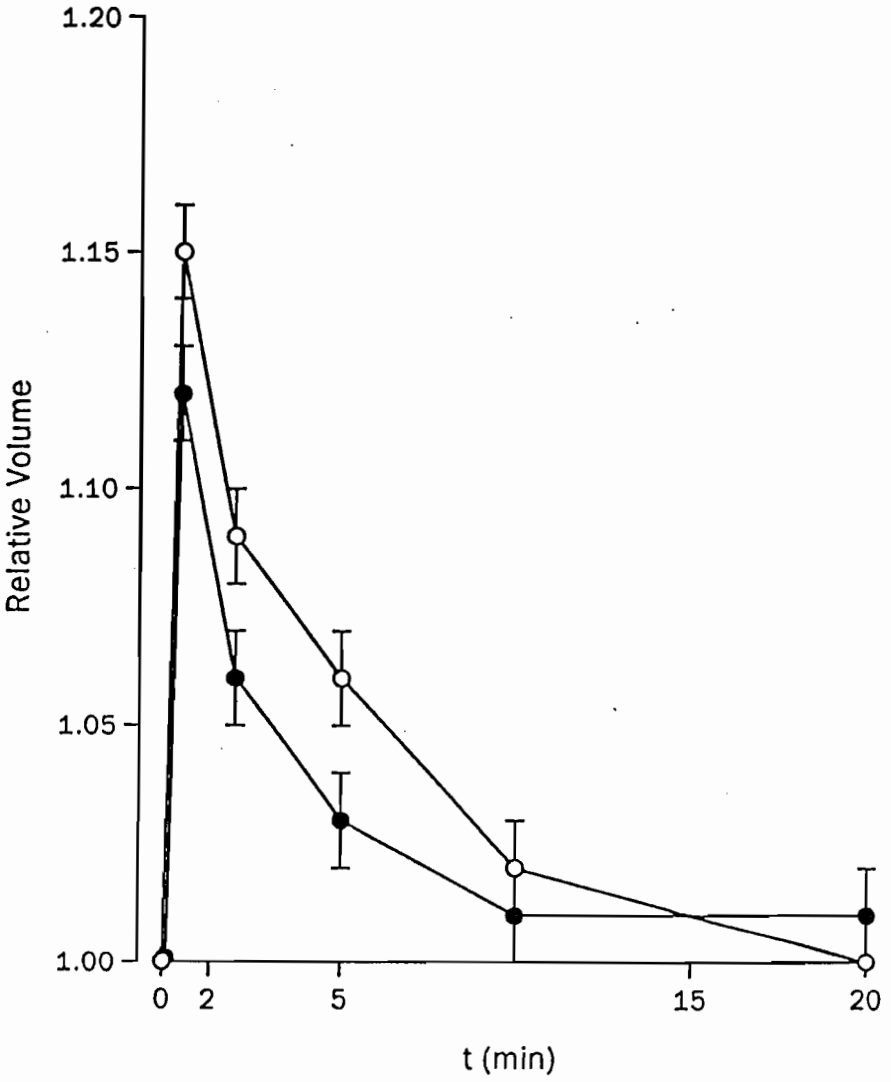

A
B
Na+- free
C
D
Na+- free
1.15
1.10
1.05
1.00
0
2
4
6
8
10
Relative Volume
Time (Minutes)

Figure Four: Effect of Na^+-free medium on RVD after greater volume increases. O, 0.70 x isotonic; ●, 0.8 x isotonic. n=6. Volume was measured electronically, expressed relative to isotonic control.

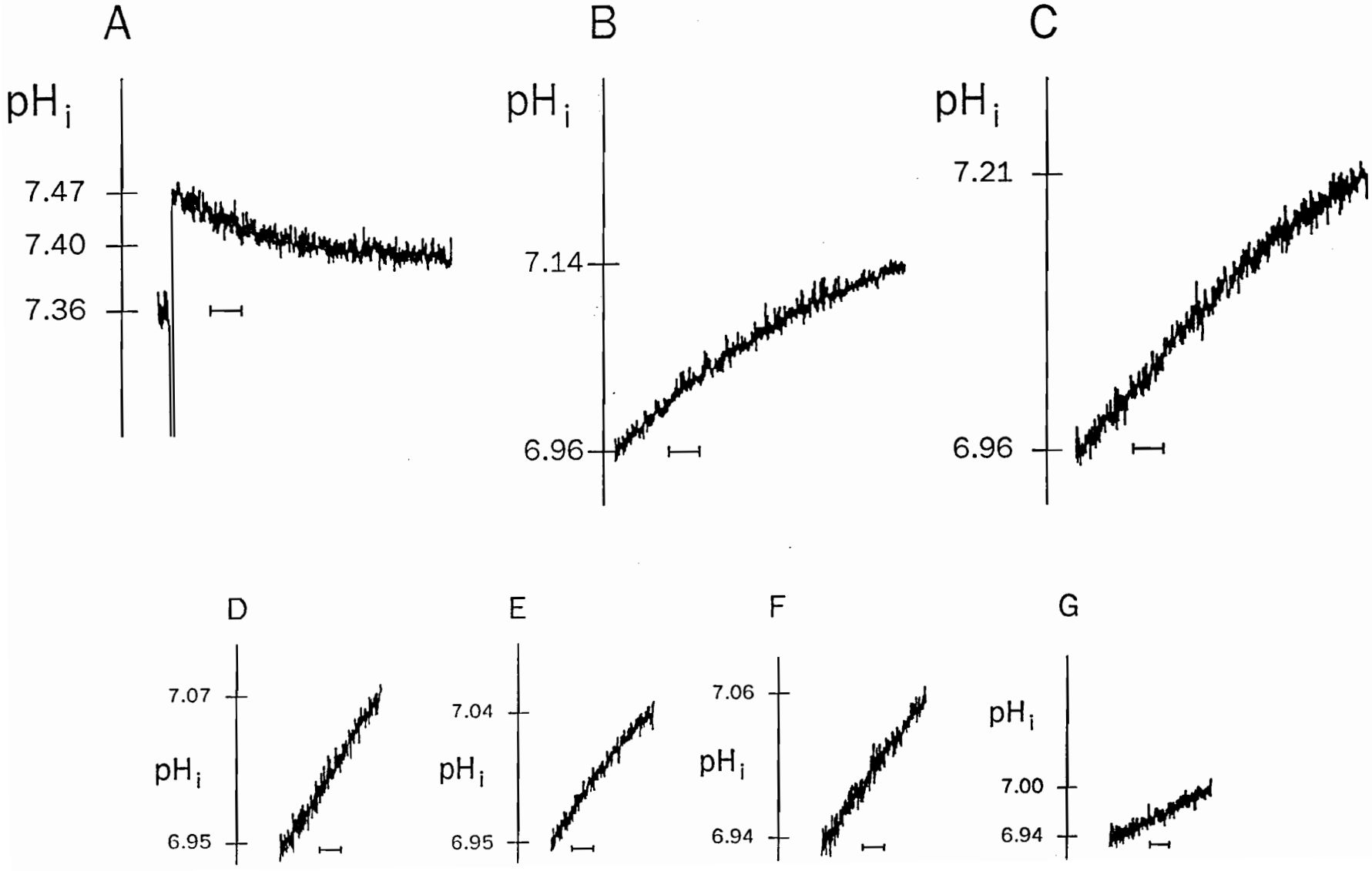

1.20
1.15
1.10
1.05
1.00
Relative Volume
0
2
5
15
20
t (min)

to block $NaKCl_2$ cotransport, the initial rate of ^{22}Na influx was attenuated by 1 μM MIA (46.8 ± 7.1 vs 29.7 ± 2.5 nmoles ^{22}Na/min/mg protein, $p<0.05$). When the cell medium was diluted 0.95 x isotonic this ^{22}Na influx rate was accelerated (73.5 ± 10.6 nmoles ^{22}Na/min/mg, $p<0.05$ vs isotonic), a response which was blocked by 1 μM MIA (37.7 ± 6.1 nmoles ^{22}Na/min/mg, $p<0.02$). Clearly, the 1 μM MIA sensitive fraction of ^{22}Na influx was increased 2 fold after a 5% hypotonic dilution.

Recovery from Intracellular Acidification

Since 0.95 x isotonic dilution caused the villus cells to acidify, we sought to determine if the activation of MIA sensitive NHE during the 5% volume increase was secondary to this acidification. The villus cells were acidified using the ammonium prepulse technique (23,24). As illustrated in Fig. 5, villus cells acidified to a pH_i of ~ 6.95 by ammonium prepulse recover. Addition of 2.5 mM NH_4Cl to these cells caused an alkalinization of 0.15 ± 0.02 pH units, n=4, Fig. 5A. The pH_i then declined (0.09 ± 0.02 pH units) over the next 5 min and did not change thereafter. When these cells were resuspended in isotonic medium (NH_4Cl-free) the time course of recovery was first order for 5 min and was ~ 41% complete in 10 min (Fig. 5B). When cells were suspended in 0.95 x isotonic medium they recovered their pH_i with a time course that was first order for 5 min and ~ 57% complete in 10 min (Fig. 5C). The rate of pH_i recovery measured over the first 5 min. was faster in 0.95 x isotonic medium than in isotonic medium ($dpH_i/dt \times 10^{-2}$ pH units min^{-1}: 3.48 ± 0.27 vs 1.59 ± 0.25, $p<0.001$, n=6). We then assessed the effect of MIA on the rate of pH_i recovery. In isotonic medium (Fig 5D), MIA slightly diminished pH_i

Figure Five: Effect of NH_4Cl prepulse on pH_i of villus cells. (A) 2.5 mM NH_4Cl, (B) pH_i recovery of acidified cells in low sodium (25mM) medium that was isotonic or (C) 5% hypotonic. Effect of N-5-methyl-isobutyl-amiloride (MIA) on pH_i recovery of acidified cells. (D) Isotonic, (E) Isotonic + MIA, 1μM, (F) 5% hypotonic, (G) 5% hypotonic + MIA. Bar indicates 1 min. Tracings are from one experiment. Comparable results obtained in five to six experiments, performed in duplicate.


A
pH$_i$
7.47
7.40
7.36
B
pH$_i$
7.14
6.96
C
pH$_i$
7.21
6.96
D
7.07
pH$_i$
6.95
E
7.04
pH$_i$
6.95
F
7.06
pH$_i$
6.94
G
pH$_i$
7.00
6.94

recovery (Fig 5E). In 0.95 x isotonic medium, MIA substantially diminished the pH_i recovery (Fig. 5F,G). The MIA sensitive rate of pH_i recovery was greater in 0.95 x isotonic medium compared with isotonic medium (1.18 ± 0.15 vs $0.43 \pm 0.03 \times 10^{-2}$ pH units min^{-1}; $p<0.001$, $n=5$). Because the MIA sensitive rate of pH_i recovery in isotonic medium was small, we speculated that identifying and controlling amiloride-insensitive sources of pH_i recovery would better resolve the MIA sensitive component of pH_i recovery from an acid load in these cells.

The effect on pH_i recovery following ammonium prepulse of bafilomycin A, a potent and selective inhibitor of type V H^+-ATPase and of Zn^{2+}, an inhibitor of H^+ conductance, are illustrated in Fig. 6. The initial rate of pH_i recovery was first-order and 18% complete (6A). Bafilomycin (100 nM) reduced the extent of pH_i recovery (ΔpH_i/2 min:64.1 ± 9.1%, $p<0.005$, Fig. 6A,C). The inclusion of Zn^{2+} (100 µM) in the presence of bafilomycin further diminished the extent of pH_i recovery (ΔpH_i/2 min: 25.4 ± 2.9%, $p<0.02$, Fig. 6B,C). Inclusion of MIA (1 µM) in the presence of Zn^{2+} and bafilomycin further diminished pH_i recovery.

Initial rate of MIA sensitive pH_i recovery from intracellular acidification in media of different tonicities

We measured the initial rate of MIA (1µM) sensitive pH_i recovery following ammonium prepulse in the presence of bafilomycin and Zn^{2+} in media of different tonicities (Fig. 7). In isotonic media, MIA (1 µM) had only a slight effect on pH_i recovery (Fig. 7A) but in 0.95 x isotonic medium, MIA completely blocked the pH_i recovery (Fig. 7B). In medium that was 0.70 x isotonic the villus cells continued to

Figure Six: Effect of Bafilomycin and Zn^{2+} on pH_i recovery from acidified cells. (A) Control, (B) Bafilomycin (100 nM), (C) Zn^{2+} (100 μM) + bafilomycin. (D) Cumulative effects of bafilomycin and bafilomycin and Zn^{2+} on pH_i recovery from acid load. *$p<0.005$ vs no additions; **$p<0.001$ vs bafilomycin. Results are mean ± SE of five experiments performed in duplicate.

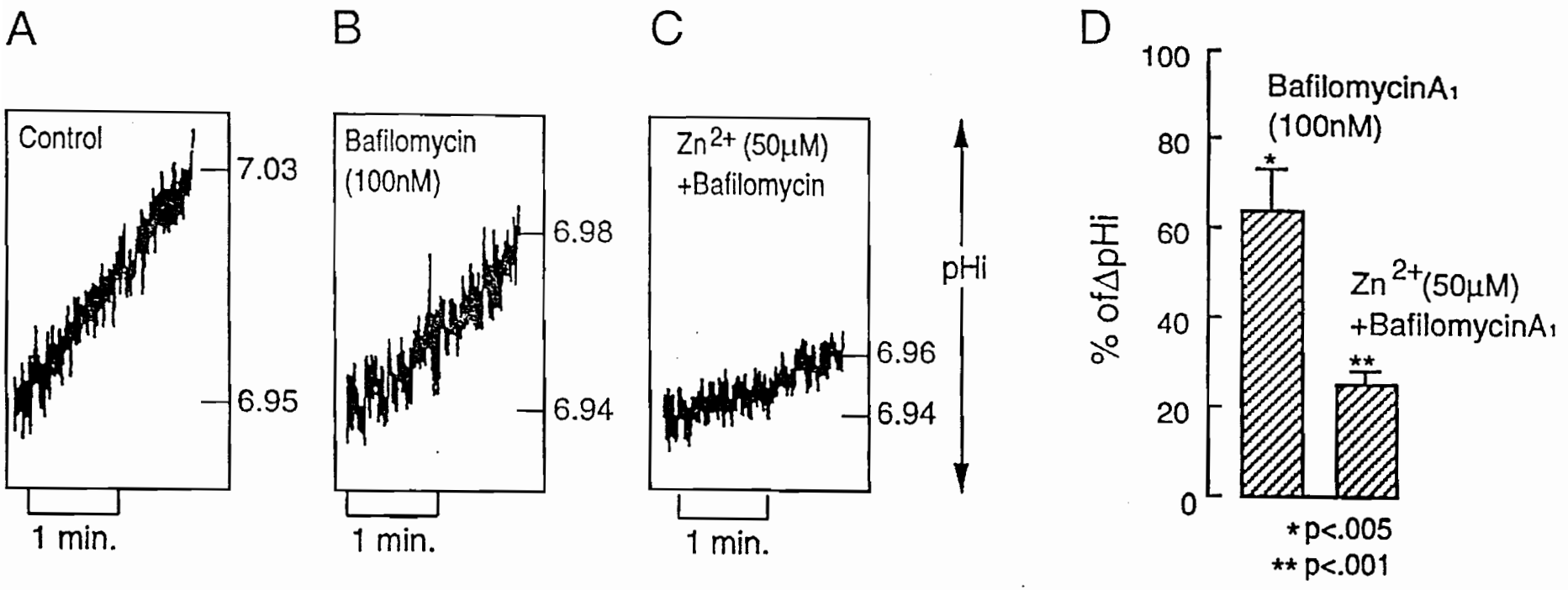

A
Control
7.03
6.95
1 min.
B
Bafilomycin
(100nM)
6.98
6.94
1 min.
C
Zn2+ (50μM)
+Bafilomycin
6.96
6.94
pHi
1 min.
D
% ofΔpHi
100
80
60
40
20
0
BafilomycinA1
(100nM)
Zn2+(50μM)
+BafilomycinA1
* p<.005
** p<.001

Figure Seven: MIA-sensitive pH_i recovery from acid load in media of different tonicities. (A) Isotonic medium containing bafilomycin and Zn^{2+} compared with MIA (1μM), bafilomycin and Zn^{2+}. (B) 0.95 x isotonic medium, bafilomycin and Zn^{2+} compared with MIA (1μM), bafilomycin and Zn^{2+}. (C) 0.70 x isotonic, bafilomycin and Zn^{2+} compared with MIA, bafilomycin and Zn^{2+}. Results of a single experiment illustrated.

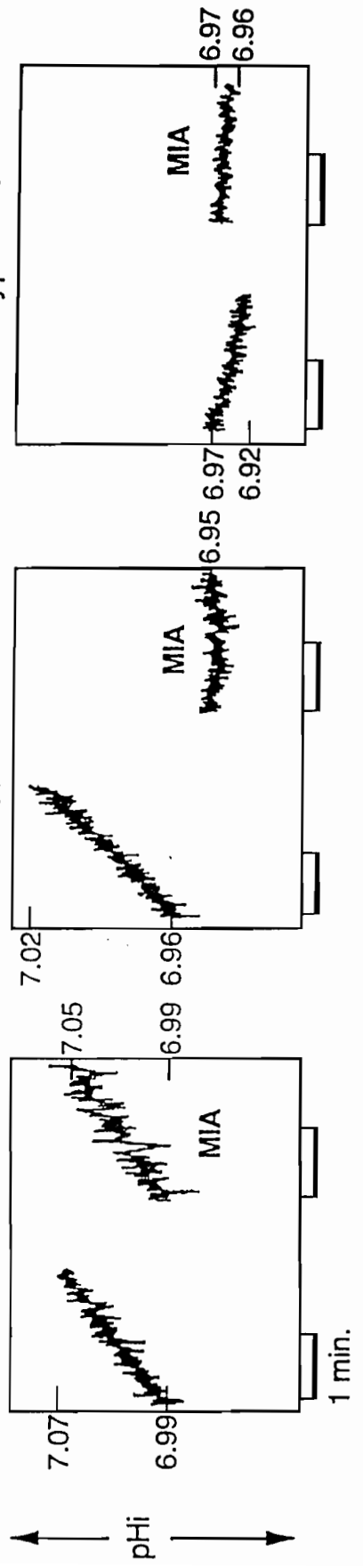

A. Isotonic
B. 5% Hypotonic
C. 30% Hypotonic
MIA
MIA
MIA
7.07
6.99
7.05
6.99
7.02
6.96
6.95
6.97
6.92
6.97
6.96
pHi
1 min.

Table 1

Initial rates of pH_i recovery from an acid load

$dpH_i/dt \times 10^{-2}$ (pH unit/min)

	Bafilomycin A_1 $+Zn^{2+}$	Bafilomycin A_1 $+ Zn^{2+}$ + 1μM MIA	MIA sensitive
Isotonic, n=7 $pH_i = 6.90 \pm 0.03$	2.06 ± 0.41	0.94 ± 0.40	1.16 ± 0.23
5% Hypotonic, n=8 $pH_i = 6.92 \pm 0.01$	3.09 ± 0.14	-0.16 ± 0.22	3.25 ± 0.26+
30% Hypotonic, n=14 $pH_i = 6.93 \pm 0.03$	-1.54 ± 0.34	-1.85 ± 0.28	0.31 ± 0.20*

Legend for Table 1

Following an NH_4^+ prepulse in RPMI medium, the rates of pH_i recovery ($dpH_i/dt \times 10^{-2}$ pH units/min) in Na^+ (25mM) 115 mM N-methyl-D-glucamine medium containing bafilomycin (100 nM) and Zn^{2+} (100 μM) or these inhibitors and N-5-methyl-isobutyl amiloride (MIA, 1 μM). Data are means ± SE of indicated number of experiments. + $p<0.001$ vs isotonic *$p<0.02$ vs isotonic.

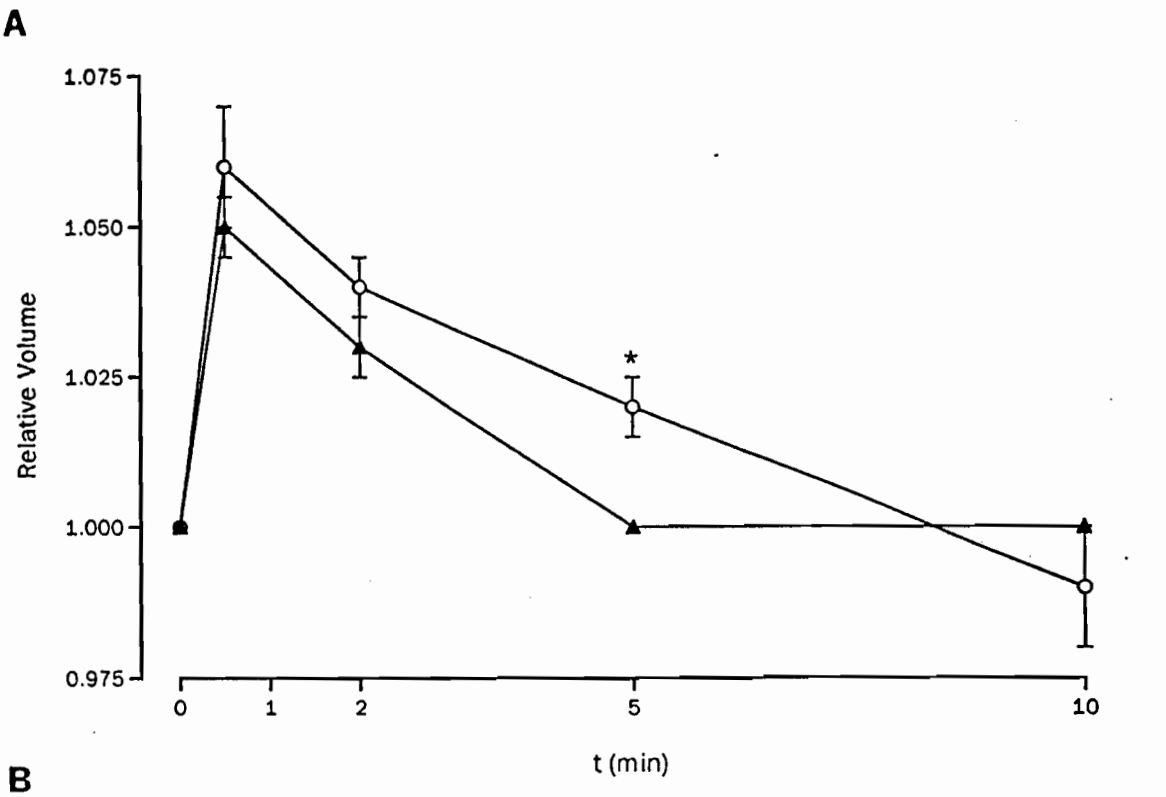
acidify; MIA attenuated this acidification (Fig. 7C). The initial rate of MIA sensitive pH_i recovery are summarized in Table 1. In all cases the starting pH_i was the same. In isotonic medium, the initial rate of MIA sensitive pH_i recovery was 1.16 ± 0.23 x 10^{-2} pH units min^{-1}. This rate was substantially increased in 0.95 x isotonic medium (3.25 ± 0.26 x 10^{-2} pH units min^{-1}, $p<0.001$). In 0.70 x isotonic medium the rate was inhibited (0.31 ± 0.20 x 10^{-2} pH units min^{-1}, $p<0.02$). Clearly, the activation of MIA sensitive Na^+/H^+ exchange by 5% hypotonic swelling was not due to intracellular acidification.

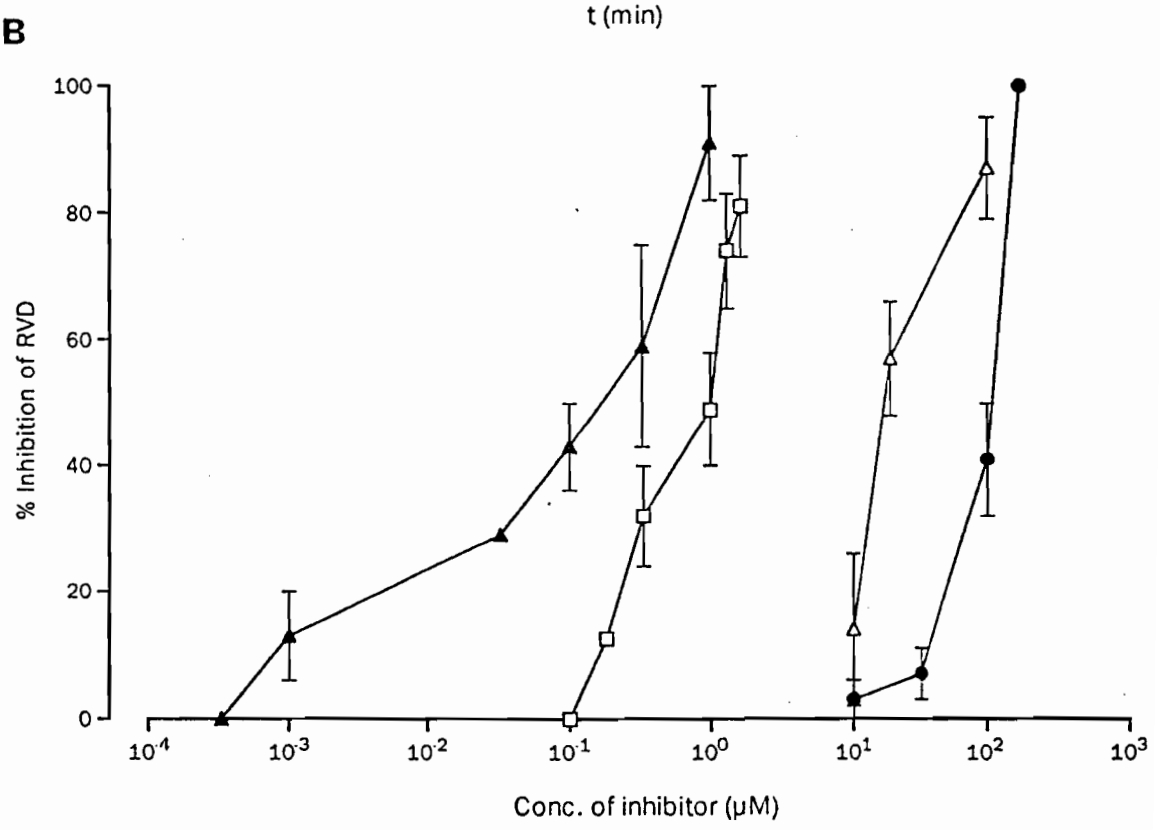
Determination of NHE Isoform activated by 5% hypotonicity

We measured the relative pharmacological sensitivities of RVD after 0.95 x isotonic dilution to several NHE inhibitors, both amiloride and non-amiloride derivatives. Cimetidine (25 μM) attenuated the rate of RVD in comparison with clonidine (50 μM) (Fig. 8A). The relative volume of cells treated with cimetidine at 5 min was greater than cells treated with clonidine (rel. vol. 1.02 ± 0.01 vs 1.00, $p<0.05$, $n=7$). Concentration-response profiles for inhibition of RVD after 0.95 x isotonic dilution are illustrated in Fig. 8B. Cimetidine was 6x more potent than clonidine. The EC_{50} of cimetidine (20 μM) and clonidine (130 μM) were greater than 5-(N,N-dimethyl) amiloride (1 μM DMA) and N-5-methyl-isobutyl-amiloride (220 nM). The order of potency of these inhibitors of the isoform of NHE activated by the 5% volume increase was:

MIA > DMA > cimetidine > clonidine (eq. 1)

Figure Eight: Effect of non-amiloride and amiloride analogues on RVD after 0.93 x isotonic dilution. (A) ▲, clonidine (50μM); O, cimetidine (25 μM), $p<0.05$, n=7. (B) ●, clonidine; Δ, cimetidine; □, N-5-dimethyl-amiloride; ▲, N-5-methyl-5-isobutyl-amiloride; n=7. Volume measured electronically, expressed relative to isotonic control.

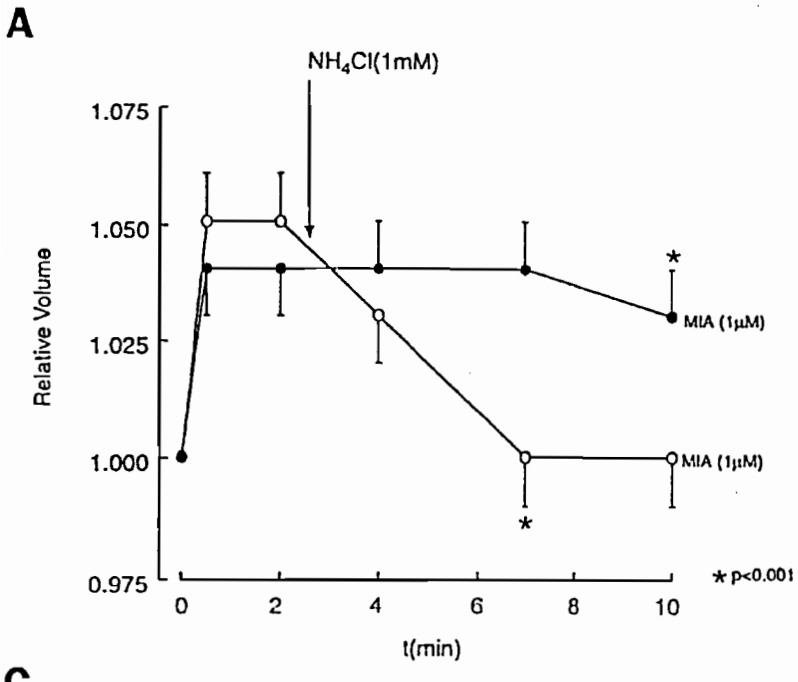

A
1.075
1.050
1.025
1.000
0.975
Relative Volume
*
0
1
2
5
10
t (min)

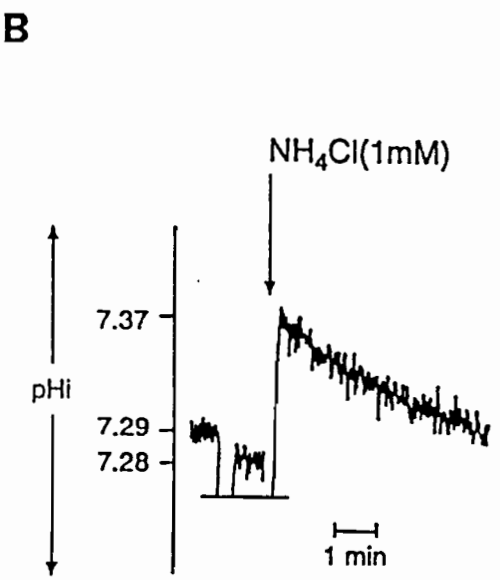

B
100
80
60
40
20
0
% Inhibition of RVD
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
Conc. of inhibitor (μM)

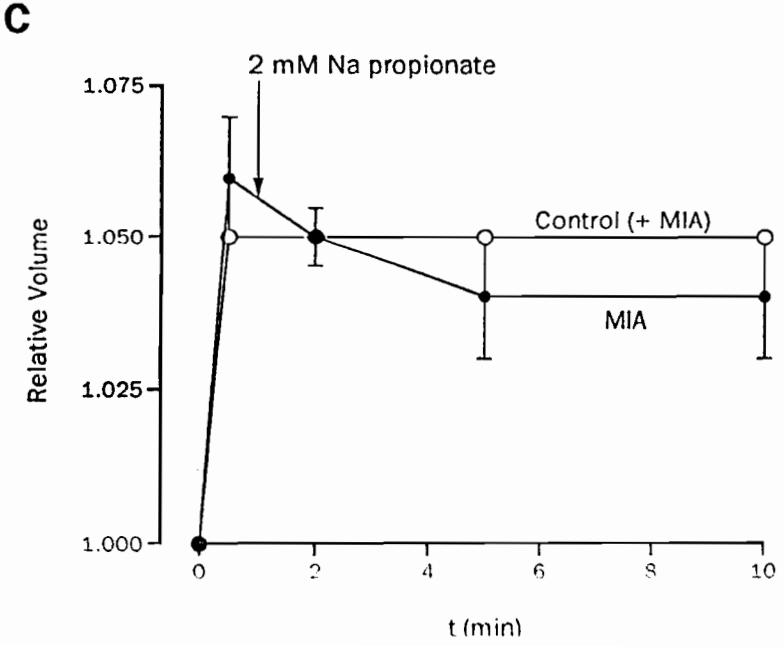
NH_4Cl induced-alkalinization allows RVD when NHE is inhibited.

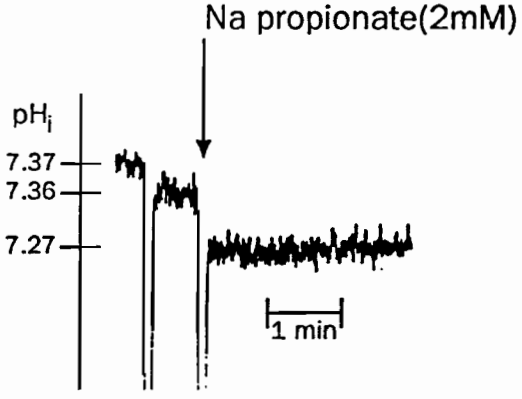
Since increasing osmolyte influx (Na^+) when the villus cells are losing K^+ and Cl^- for RVD seems counterintuitive, we measured volume and ΔpH_i in cells hypotonically diluted 7% in the presence of MIA (1μM) and following addition of 1 mM NH_4Cl (Fig. 9). As previously observed, MIA (1μM) prevented RVD after cell swelling following 7% hypotonic dilution (Fig. 9A). Addition of 1mM NH_4Cl to the swollen cells caused RVD in the presence of MIA. Within 2 min of the addition of NH_4Cl these cells started to shrink and the RVD was complete in the next 3 min (rel vol.: 1.03 ± .01 vs 1.00 ± .01, $p<0.001$). Immediately after the addition of 1 mM NH_4Cl the villus cells alkalinized (Fig 9B). This alkalinization (0.086 ± 0.010 pH units, n=15) was no different than that measured in these cells following 5% hypotonic dilution (Fig 2). We then acidified the pH_i of comparably treated cells to show the converse of the alkalinization experiment (Fig. 9C, D). Na^+ propionate (2 mM) added to cells hypotonically diluted 7% in the presence of MIA had no effect on the inhibited RVD (Fig. 9C). Addition of Na^+ propionate to these cells caused an acidification (0.086 ± 0.016 pH units, n=10) which remained stable for the next 5 min. of the experiment (Fig. 9D). This experiment suggests that it is alkalinization of pH_i caused by the NHE activated by a modest cell swelling which signals the ion conductances for the subsequent volume regulation.

Figure Nine: NH_4Cl induced-alkalinization allows RVD when NHE-1 is inhibited. (A) RVD blocked by MIA is bypassed with NH_4Cl (1mM) addition. ●, MIA (1μM), 0.93 x isotonic dilution. O, NH_4Cl (1mM) added immediately after 2 min volume assessment made; MIA (1μM), 0.93 x isotonic. Volume measured electronically, expressed relative to isotonic control. *$p<0.001$, MIA vs MIA + NH_4Cl at 7 and 10 min. (B) pH_i tracing of NH_4Cl (1mM) addition. Cells were diluted 0.93 x isotonic in the presence of MIA (1μM) 1 min later NH_4Cl was added. Tracing, corrected for dilutional artifact, is from a single representative experiment. Net alkalinization by this manoeuvre was 0.086 ± 0.010 pH units, n=15. (C) Na^+ propionate (2 mM) added to MIA treated cells. (D) pH_i tracing of Na^+ propionate (2 mM) addition to cells treated as in (C). Tracing is from one representative experiment.


A
NH4Cl(1mM)
1.075
1.050
1.025
1.000
0.975
Relative Volume
MIA (1μM)
MIA (1μM)
* p<0.001
0
2
4
6
8
10
t(min)


B
NH4Cl(1mM)
pHi
7.37
7.29
7.28
1 min


C
2 mM Na propionate
1.075
1.050
1.025
1.000
Relative Volume
Control (+ MIA)
MIA
0
2
4
6
8
10
t (min)


D
Na propionate(2mM)
pHi
7.37
7.36
7.27
1 min

DISCUSSION

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

The RVD following modest volume increase was prevented by the non-amiloride derivatives cimetidine and clonidine, with cimetidine being six times more potent than clonidine. This observation provides strong evidence that NHE-1 is the isoform activated by the 5 to 7% volume increase. Studies using transfectants of NHE isoforms have shown that clonidine is more potent than cimetidine inhibiting NHE-2 and NHE-3, while only with NHE-1 is this order of potency reversed (25,26). K^+, a weak competitive inhibitor of NHE-1 but not NHE-2 (26), prevented the N-5-methyl-isobutyl-amiloride (MIA) sensitive rate of pH_i recovery from an acid load, strongly supporting the view that the isoform responsible for these pH_i effects is NHE-1. Jejunal villus epithelial cells possess three isoforms of NHE (17,27,28).

NHE-3 and NHE-2 are found on the apical membrane, and are both more sensitive to clonidine than cimetidine, while NHE-1, which is more sensitive to cimetidine than clonidine, has been localized to the basolateral membrane of villus cells (27,28,29). We found that MIA was more potent than dimethyl-amiloride in preventing RVD after the modest 5 to 7% volume increase, but this hierarchy is the same for NHE-1, -2 and -3 (26). After a 5% volume increase, but not a 15% volume increase, the RVD, the alkalinization of pH_i, the increased ^{22}Na influx and recovery of pH_i from an acid load were all prevented by a low concentration of the N-5-alkyl amiloride derivative. As cimetidine was more potent than clonidine in preventing this RVD, when taken together, our data strongly suggest that NHE-1 is the isoform of NHE activated during the modest, 5-7% volume increase of the villus cells.

The fact that the MIA sensitive rate of pH_i recovery from an acid load was accelerated in cells suspended in a medium 0.95 x isotonic but inhibited in cells suspended in the `standard' hypotonic medium (0.70 x isotonic) suggested that activation of NHE-1 is not directly related to intracellular acidification. The well documented kinetic asymmetry of NHE-1 in symmetrical cells (12), evidenced by a sigmoidal relationship between the Na^+/H^+ exchange rate and internal H^+ concentration suggested such cooperativity was because of an intracellular H^+ modifier site, distinct from the H^+ transport site, and that regulation of NHE occurs via changes in the affinity for intracellular H^+ of this internal H^+ modifier site (12,30). We measured differences in the rate of pH_i recovery from an acid load after resuspending the villus cells in media of different tonicities but with $[Na^+]_o$ reduced to 25 mM. We used these conditions because others using A6 cells, an epithelial cell line which exhibits both apical and basolateral NHE (31), have observed that

pH_i changes due to the basolateral NHE, were greatest at low Na^+ concentrations (32). Our findings of a substantial increase in pH_i recovery in 0.95 x isotonic medium compared with isotonic low Na medium are in accord with these data from A6 cells. The inhibition of MIA sensitive pH_i recovery in medium that was 0.70 x isotonic but with the same Na^+ concentration is consistent with reports of the effect of substantial hypotonicity on NHE in symmetrical cells. In nominally HCO_3 free medium, after acid loading, osteosarcoma cell suspensions undergo amiloride sensitive pH_i changes which are diminished in medium that is 0.70 x isotonic (9). Kinetic analysis of these data demonstrated that inhibition of the exchanger was due to decreased V_{max} without a change in apparent affinity for H^+ or Na^+. Single cell analysis of ΔpH_i after 0.7 x hypotonic dilution confirmed that NHE was inhibited following cell swelling (10). We have clearly shown that the MIA sensitive pH_i recovery from an acid load increased in medium that was 0.95 x isotonic but decreased in 0.7 x isotonic compared with isotonic controls. We conclude that the activation of basolateral NHE-1 during modest 5 to 7% volume increase in the villus cells is not a consequence of the intracellular acidification normally observed after cell swelling.

Approximately 75% of the pH_i recovery from a moderate acid load in the villus cells was insensitive to the N-5-alkyl amiloride derivative. Our finding that bafilomycin inhibited 36% of the pH_i recovery, suggests that a V type H^+-ATPase contributes to pH_i homeostasis in these cells (33). This atpase, which has been localized to the apical membrane of urinary bladder epithelial cells (34) and the plasma membrane of peritoneal macrophages (35), has been shown to contribute to pH_i recovery from acid loads in the presence of amiloride in both peritoneal (20)

and alveolar macrophages (36). We also observed that Zn^{2+}, in the presence of this selective inhibitor of vacuolar (v) H^+-ATPase, further reduced by ~39% the pH_i recovery of the villus cells. The concentration of Zn^{2+} used in our experiments has been shown by others to block H^+ conductance in snail neurons (37) and human granulocytes (38). Furthermore, unequivocal results using transfectants of NHE-1 have shown that H^+ conductance which is Zn^{2+}-sensitive, may be dissociated from NHE activity and that substantial alkalinizations of pH_i due to NHE-1 still occur in the presence of $ZnCl_2$ (39). As such, the sensitivities of pH_i recovery from an acid load to bafilomycin and to Zn^{2+} suggest both H^+ conductance and a V type-ATPase substantially contribute to pH_i homeostasis in villus cells.

Cellular acidification after cell swelling because of hypotonic dilution (0.7 to 0.5 x isotonic) has been observed in several symmetrical cell types. The source of acidification has been speculated to be conductive OH^- efflux through volume-activated Cl^- channels (10), increased glycolytic metabolic activity (8) or inhibited NHE activity (9). We found that cell swelling of 15% following this `standard' hypotonic dilution inhibited NHE activity. Clearly, several mechanisms contribute to swelling-induced cellular acidification since modest 5 to 7% volume increases similar to swelling caused by Na^+ solute absorption cause villus cells to acidify as well as activate NHE-1. Far from being a `housekeeping' function, the activation of NHE-1 may be an essential requirement for RVD following `physiological' volume increases, when these cells swell during Na^+-solute absorption.

Alkalinizing the pH_i of cells swollen 5% in the presence of MIA by-passed inhibition and allowed complete RVD, whereas acidifying the pH_i had no

effect on the inhibited volume reduction. The extent of this alkalinization, induced by NH_4Cl addition, was comparable to that observed in cells swollen after 0.95 x isotonic dilution. This finding suggests that Na^+ influx resulting from the activated NHE-1 is osmotically neutral and that it is the change in pH_i which is a determinant of the osmolyte loss (K^+ and Cl^-) required for volume regulation. Previously we reported that RVD following swelling because of the uptake of D-glucose was sensitive to the high conductance Ca^{2+}activated (maxi-K) K^+ channel blocker charybdotoxin, while RVD following a greater swelling of 15% caused by the standard 0.70 x isotonic dilution was insensitive to the toxin (3). Since the calcium gating of charybdotoxin sensitive K^+ conductance is exquisitely sensitive to alkaline pH_i (40), the activation of NHE-1 during the modest 5 to 7% volume increase may serve as the source of the required alkalinization for Ca^{2+} gating of the charybdotoxin sensitive K^+ loss. Because villus cells acidify as they swell 15% of their isotonic volume, we speculate that a different K^+ conductance is activated for RVD following larger, `non-physiological', volume increases. As villus cells swell, the extent of that swelling is a key determinant of changes in pH_i, which in turn serve to signal the subsequent volume regulation.

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A modest (5 to 7%) volume increase of the villus epithelial cells results in the activation of amiloride-sensitive Na^+/H^+ exchange (NHE). The subsequent volume recovery (RVD) absolutely requires a transient alkalinization of intracellular pH (pH_i) which is due to the activated NHE. Because the rate of amiloride-sensitive pH_i recovery from an acid load was faster in 5% hypotonic medium compared with isotonic medium, we assessed the role of heterotrimeric GTP-binding proteins (G-proteins) in mediating this activation of NHE. Experiments were designed to answer the following questions: Are the alkalinization of pH_i and RVD blocked in villus cells treated with GDPβS, a G protein antagonist? In isotonic medium, does treating these cells with a G protein agonist, GTPγS, stimulate amiloride-sensitive pH_i changes? Is the amiloride-sensitive rate of pH_i recovery from an acid load of cells treated with GTPγS in isotonic medium greater than untreated cells, but equivalent to untreated cells in 5% hypotonic medium? To answer these questions we loaded the villus cells with photoactivatable G protein agonists or antagonists using electropermeabilization. Once the cells had resealed, we measured pH_i changes and volume responses.

Chapter Three

G protein mediated activation of Na^+/H^+ exchange required for RVD after modest `physiological' volume increases in Jejunal Villus enterocytes

Abstract

The uptake of Na^+ cotransported nutrients like L-alanine or D-glucose by villus epithelial cells results in a modest, 5 to 7% increase in volume which is followed by a Regulatory Volume Decrease (RVD) due to the activation of K^+ and Cl^- channels. We duplicated this physiological volume increase by suspending the villus cells in medium that was 5% hypotonic and measured changes in cell volume with electronic cell sizing and intracellular pH (pH_i) with fluorescent spectroscopy of villus cells in suspension loaded with 2', 7'-bis (carboxyethyl)5-(6)-carboxyfluorescein (BCECF). Following suspension in medium that was 5% hypotonic, the villus cells exercised RVD that was prevented ($p<0.001$) by 1 μM N-5-methyl-5-isobutyl amiloride (MIA), an inhibitor of the NHE-1 isoform of Na^+/H^+ exchange in these cells. The pH_i alkalinized 0.06 ± 0.01 pH units which was prevented ($p<0.001$) by MIA. In villus cells loaded by electropermeabilization with an antagonist of G protein activation, 1 mM guanosine 5'-0-2-thiodiphosphate (GDPβS), the ΔpH_i was blocked ($p<0.001$) as was RVD ($p<0.001$). In isotonic medium, villus cells loaded by electropermeabilization with `caged' 100μM guanosine 5'-0-(3-thiotriphosphate),3-S-(1-(4,5-dimethoxy-2-nitrophenyl)ethyl)thioester(DMNPE-GTPγS), which causes prolonged activation of G proteins, after photolysis caused alkalinization (ΔpH_i/5 min: 0.105 ± 0.025). This alkalinization was blocked by 1 μM MIA ($p<0.005$) or by loading cells with 1mM GDPβS ($p<0.005$). The MIA sensitive rate of pH_i recovery from an acid load in isotonic medium was increased in cells loaded with DMNPE-GTPγS after photolysis ($p<0.005$) and inhibited ($p<0.001$) by 1mM GDPβS. The MIA sensitive rate of pH_i

recovery from an acid load in 5% hypotonic medium was equal to the rate in isotonic medium of cells loaded with GTPγS. When RVD was prevented in cells loaded with GDPβS, addition of NH_4Cl caused the pH_i to alkalinize and allowed RVD to proceed. Dihydrocytochalasin B (10μM) prevented RVD after 5% swelling and the ΔpH_i ($p<0.001$). Our data suggest the hypotonic activation of NHE-1 which duplicates `physiological' volume increases is mediated by a G protein and that intact actin microfilaments are required to transduce this response.

Index terms:

G protein, NHE, volume decrease, enterocyte

Introduction

A modest volume increase of 5 to 7% of a jejunal villus epithelial cell stimulates Na^+/H^+ exchange (NHE) activity (26,27,28). The extent of this volume increase is physiologically relevant because it duplicates the extent to which these cells swell during absorption of Na^+-solute (22,24,25). The Regulatory Volume Decrease (RVD) following this modest volume increase, which is due to the activation of K^+ and Cl^- channels, absolutely requires a Na^+-dependent, amiloride-sensitive alkalinization of intracellular pH (pH_i) for volume recovery (26,28). This pathway differs from volume increases of 15 to 20% after `standard' hypotonic dilution (0.70 to 0.50 x isotonic) where NHE activity is inhibited (19,28). In this report, using suspended jejunal villus epithelial cells exposed to a slight hypotonic challenge (0.95 x isotonic) to duplicate the volume increase occurring because of Na^+-solute absorption (5 to 7% swelling) we show a precise relationship between activation of GTP-binding or G proteins and the activation of NHE required for the subsequent RVD. We used electropermeabilized villus cells loaded with photoactivatable GTPγS to cause prolonged activation of a G protein and GDPβS as a G protein antagonist, and measured pH_i and cell volume changes. Our data suggest that activation of NHE by modest volume increases is mediated by a G protein and that intact microfilaments are required to transduce this response.

Materials and Methods

Villus cell isolation and volume determination

Villus cells were isolated from segments of adult male (200-300g) guinea pig jejunum by mechanical vibration as previously described (21). Isolated cells were resuspended at 0.8-1.5 x 10^6 cells/mL in RPMI-1640 medium (without HCO_3) containing bovine serume albumin (type V) at 1 mg/mL and 20 mM NaHepes pH 7.3 at 37° C. Viability, assessed by trypan blue exclusion was 85%, 3 hr. after suspension in medium. Cell volume was measured using a Coulter Counter (model Zm) with an attached Channelyzer (C-256) as previously described (22,24,25). Villus cell volume measured electronically over a range of tonicities correlated (τ = 0.967) with direct measurements of cell water (23). Relative cell volume was calculated as a ratio of the volume under study conditions to the volume under basal conditions in isotonic medium.

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). Low Na^+ medium contained 25 mM NaCl with 115 mM of the chloride salt of N-methyl-D-glucamine ($NMDG^+$). K^+ rich medium had NaCl isosmotically replaced by KCl. Permeabilization medium contained (in mM): 140 KCl, 1 $MgCl_2$, 1 ATP (K^+ salt), 10 D-glucose, 10 Hepes, 9 $NMDG^+$, 1 EGTA, 0.193 $CaCl_2$ and bovine serum albumin (fraction V) at 0.1 mg/mL (pH 7.0).

pH_i Measurement and Manipulation

For the fluormetric determination of pH_i villus cell suspensions (1×10^6 cells/mL in Hepes-RPMI) were loaded with BCECF by incubation with the parent acetoxymethylester (3.7μM) for 15 min at 37°C. After washing, 0.5 to 0.8×10^6 cells were used for fluorescence determination in 2 ml of the indicated medium using a Hitachi F-4000 spectrofluormeter with excitation at 495 nm and emission at 525 nm using 5 and 10 nm slits, respectively. Acid loading was accomplished by preincubation of 10^6 cells/mL for 5 min in RPMI medium containing 2.5 mM NH_4Cl at 37°C, followed by sedimentation and resuspension in 2mL of the indicated NH_4^+-free medium. For experiments using acid-loaded cells, loading with NH_4^+ and BCECF was performed simultaneously. The 5-N-methyl-N-isobutyl-amiloride (MIA) sensitive rate of pH_i recovery was the difference between pH_i recovery in the presence of bafilomycin (100 nM) and Zn^{2+} (50μM), and pH_i recovery in the presence of these inhibitors and 1μM MIA, as previously described in detail (28). The resting pH_i of villus cells in Hepes buffered RPMI-1640 medium (nominally HCO_3-free) was 7.39 ± 0.04, n=45. Calibration was performed in K^+ rich medium using nigericin (36) and a quench correction factor as described (13).

Permeabilization and Loading of DMNPE-GTPγS

We modified a procedure previously reported for human neutrophils (14). In preliminary experiments we determined the conditions to effect dielectric breakdown of the apical/basolateral membranes of villus cells in suspension by measuring cell volume response electronically. As expected from the Donnan effect (14), permeabilization leads to cell swelling. We determined conditions for

resealing cell membranes after permeabilization by resuspending cells in RPMI-1640 medium. Cells were then suspended in Na^+ (140mM) medium that was subsequently diluted 0.5 x isotonic to cause cell swelling. If the membrane had resealed, this swelling was followed by a Regulatory Volume Decrease (RVD, 23); if the membrane was still permeable this initial cell swelling was followed by continued cell swelling. Therefore, appropriate permeabilization was assumed if after electrical discharge, the cells swelled in isotonic medium; appropriate resealing of these cells was assumed if we observed a complete RVD response after suspension in 0.5 x isotonic medium.

Villus cells were permeabilized immediately before use. An aliquot of 10^7 cells was washed once and resuspended in 0.8 mL of ice-cold permeabilization medium containing 100 μM DMNPE-GTPγS. The suspension was transferred to a covered Bio-Rad Pulser cuvette and subjected to three discharges of 0.9 kV/cm from a 25-microfarad capacitor, using an IBI gene Zapper 450/2500 System with a time constant of 0.6 to 0.7 ms. These cells were centrifuged (200 x g) and resuspended in 5 mls of NaHepes buffered RPMI-1640 medium in plastic tubes in the dark; they were allowed to re-seal for 15 min at 37°C, after which they were loaded with BCECF by incubation with 3 μM BCECF-AM for an additional 10 min. Aliquots of $3x10^6$ cells/mL were washed and resuspended in the appropriate buffer and subjected to photolysis for 60s using a Hitachi F-4000 spectrofluorimeter with excitation at 360 nm using a 10 nm slit width in a constantly stirring thermostated cuvette. Changes in pH_i were then determined fluorimetrically as described above.

In some experiments villus cells were permeabilized in medium containing 100μM DMNPE-GTPγS and 1 mM GDPβS or GDPβS alone.

Chemicals

The acetoxymethyl ester of 2',7', bis (carboxyethyl) 5-(6)-carboxyfluorescein (BCECF) and guanosine-5'-0-(3-thiotriphosphate),3-S-(1-(4,5 dimethoxy-2-nitrophenyl)ethyl)thioester (DMNPE-GTPγS) were obtained from Molecular Probes (Eugene, OR). 5-N-methyl (-N-isobutyl amiloride (MIA) was from Research Biochemicals (Natick, MA) and $NMDG^+$ from Aldrich (Milwaukee, WI). Nigericin, EGTA and GDPβS were from Sigma Chemical (St. Louis, Mo), and RPMI-1640 (10x) medium was from GIBCO/BRL (Burlington, Ont.). We purchased bafilomycin A, from Dr. K. Altendorf (Universitat Osnabruck, Germany).

Statistics

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

Results

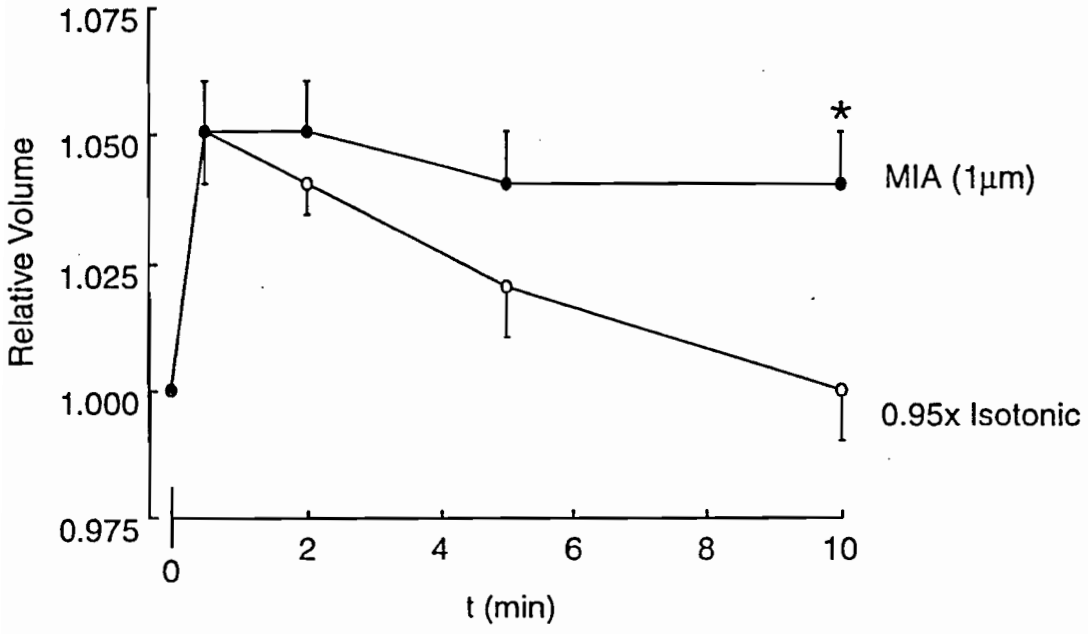
Effect of 5% volume increase on pH_i and RVD.

Villus cells were suspended in 0.95 x isotonic buffer, because at that concentration we had found that cells swelled to a size comparable to the swelling occuring during Na^+-solute absorption. The pH_i and volume responses are illustrated in Fig. 1. After swelling, the cells demonstrated complete Regulatory Volume Decrease (RVD). This RVD was prevented by N-5-methyl-5-isobutyl amiloride (MIA, 1μM) indicating that NHE activation was required for volume reduction. The final relative volume of the cells was larger in the presence of MIA compared with untreated controls (1.04 ± .01 vs 1.00 ± 0.01, $p<0.001$, Fig 1A). Furthermore, the pH_i of these cells alkalinized (ΔpH_i/3 min: 0.06 ± 0.01 pH units, Fig 1B), a response that was blocked by the amiloride derivative ($p<0.001$). Then villus cells in suspension were loaded with 1 mM GDPβS by electropermeabilization and allowed to reseal. When hypotonically diluted 5% these cells did not alkalinize, they acidified. This change in pH_i was in the opposite direction compared with untreated controls (ΔpH_i/3 min: -0.03 ± 0.01 vs 0.06 ± .01 pH units, $p<0.001$, Fig 1B). Because GDPβS will function as a G protein antagonist, these data suggest that activation of NHE was mediated by a G protein.

Effect of GTPγS on isotonic NHE activity.

To study a putative role of a GTP-binding protein in the activation of NHE in the villus cells, we first determined the effect of GTPγS on pH_i in isotonic (140mM Na^+) medium (Fig. 2). After loading with 100 μM DMNPE-GTPγS by electroporation followed by resealing of the membrane, the villus cells were acidic.

Figure One: Effect of N-5-methyl-N-isobutyl amiloride (MIA) on RVD and $_{\Delta}pH_i$ after 0.95 x isotonic dilution. (A) O, 0.95 x isotonic; ●, MIA (1μM) in 0.95 x isotonic, *p<0.001 vs control, n=6. (B) $_{\Delta}pH_i$/3 min. *p<0.001 vs 0.95 x isotonic control, n=6. Volume measured electronically, expressed relative to isotonic controls. Cells loaded with GDPβS by electroporation followed by resealing and pH_i assessed as described in Methods.


A
1.075
1.050
1.025
1.000
0.975
Relative Volume
0
2
4
6
8
10
t (min)
*
MIA (1μm)
0.95x Isotonic

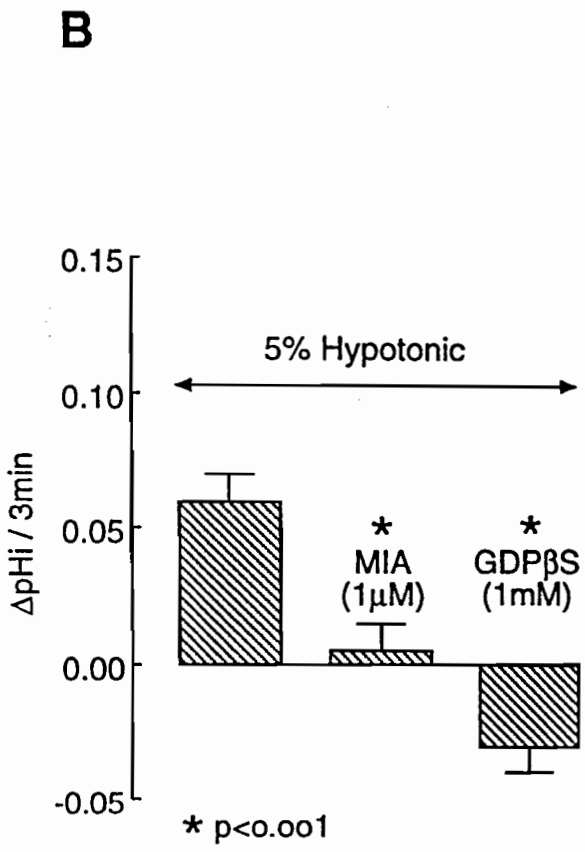
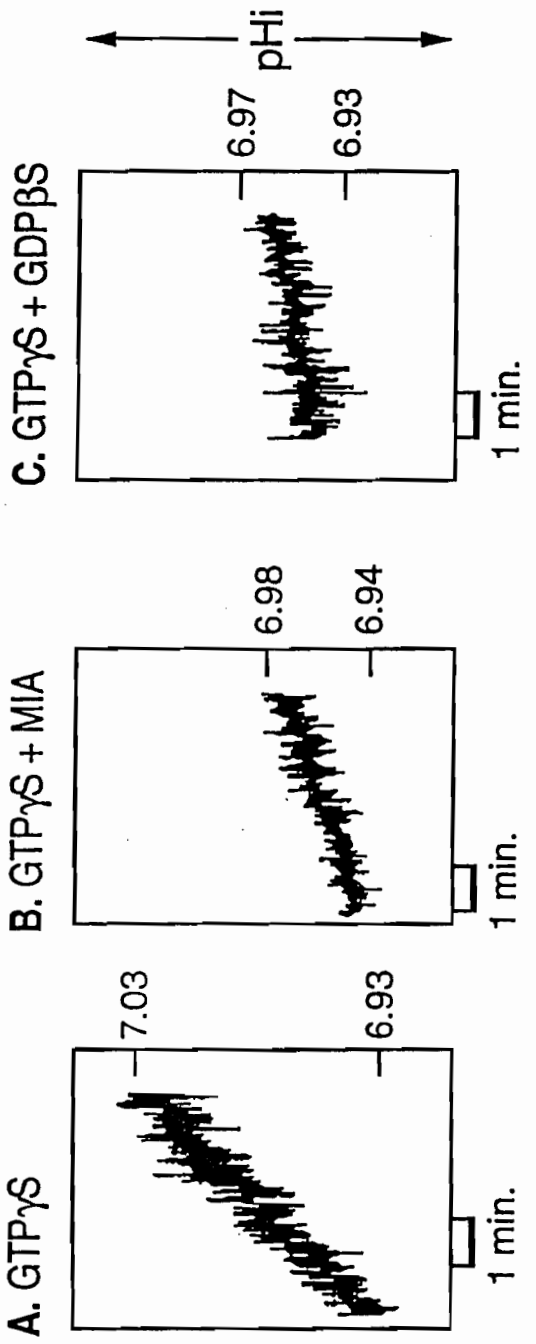
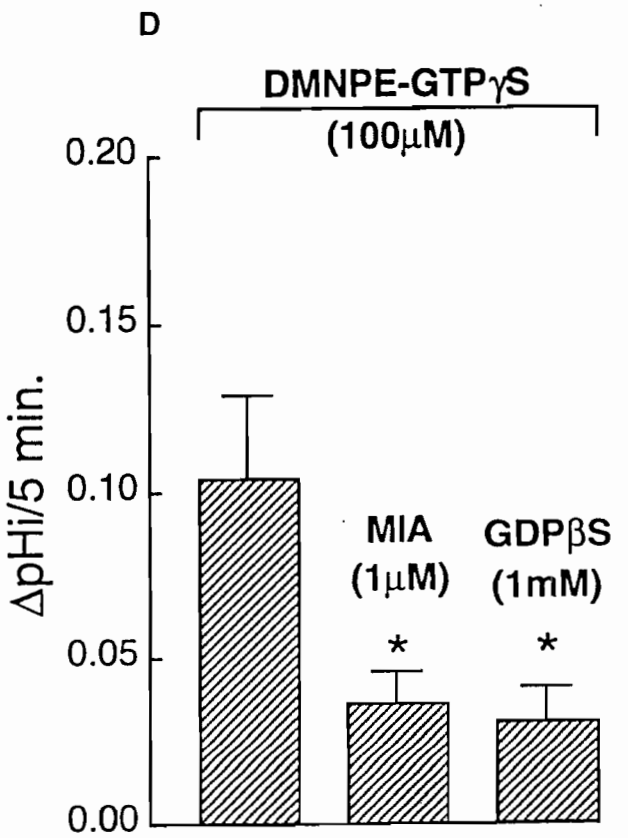

B
0.15
0.10
0.05
0.00
-0.05
ΔpHi / 3min
5% Hypotonic
*
MIA
(1μM)
*
GDPβS
(1mM)
* p<o.oo1

Figure Two: Effect of GTPγS on isotonic pH_i of villus cells. After loading by electropermeabilization with 100μM DMNPE-GTPγS and following membrane re-sealing, GTPγS was photoactivated for 60S, then changes in pH_i were determined in the absence (A) or presence (B) of 1μM N-5-methyl-isobutyl amiloride (MIA). pH_i changes of cells loaded with 100 μM DMNPE-GTPγS and 1 mM GDPβS after photoactivation (C). Time scale represents 1 min, after photoactivaiton. Data are representative of five experiments, performed in duplicate, which are summarized as means ± SE (D). *$p<0.005$ compared with GTPγS alone.


A. GTPγS
7.03
6.93
1 min.
B. GTPγS + MIA
6.98
6.94
1 min.
C. GTPγS + GDPβS
6.97
6.93
pHi
1 min.


D
DMNPE-GTPγS
(100μM)
0.20
0.15
0.10
0.05
0.00
ΔpHi/5 min.
MIA
(1μM)
GDPβS
(1mM)
*
*

Following photoactivation of the caged GTPγS, the pH_i alkalinized (Fig 2A); this alkalinization was attenuated by 1μM MIA (Fig. 2B). Measured over 5 min, photoactivation caused a ΔpH_i that was inhibited by 1 μM MIA (ΔpH_i/5 min: 0.105 ± 0.025 vs 0.040 ± 0.010 pH units, $p<0.005$, n=5, Fig. 2D). Staurosporine (10nM), a potent inhibitor of protein kinase C in these cells (24) had no effect on the ΔpH_i stimulated by photoactivation (0.115 pH units, n=3). We also assessed the effect of the G protein antagonist, GDPβS on these ΔpH_i. Villus cells were loaded with 1mM GDPβS together with `caged' GTPγS by electroporation; after photoactivation of `caged' GTPγS these cells had an attenuated alkalinization (Fig. 2C). GDPβS inhibited the ΔpH_i stimulated by GTPγS (ΔpH_i/5 min: 0.035 ± 0.010 pH units, $p<0.005$, n=5, Fig. 2D). There was no difference in the incremental alkalinization caused by GTPγS in the presence of 1μM MIA and that observed in the presence of GDPβS, but the absence of MIA (Fig 2D). These results suggest that NHE, which is sensitive to low concentrations of MIA, may be activated by a G protein. We also assessed whether DMNPE-GTPγS loaded cells changed volume under basal isotonic conditions (Fig. 3). Following photoactivation, an aliquot of cells was added to isotonic medium and compared with comparably treated cells in medium containing 1 μM MIA. Within 10 min., the GTPγS loaded cells were larger than treated but unloaded cells (final rel. vol. 1.05 ± 0.01 vs 1.0 ± 0.01,$p<0.05$, Fig. 3). The amiloride derivative blocked the volume increase of GTPγS loaded cells (final rel. vol. 0.95 ± 0.01, $p<0.01$, n=5, Fig. 3).

Figure Three: Effect of GTPγS on isotonic volume of villus cells. After loading by electropermeabilization with 100 μM DMNPE-GTPγS and following membrane re-sealing, GTPγS was photoactivated for 60S, and cells were resuspended in Na^+ (140 mM) medium and volume was assessed in the absence (▲) or presence (O) of 1 μM MIA. Data are representative of five experiments performed in duplicate. Volume measured electronically, expressed relative to unloaded, electropermeabilized cells. *$p<0.01$ compared with GTPγS alone.

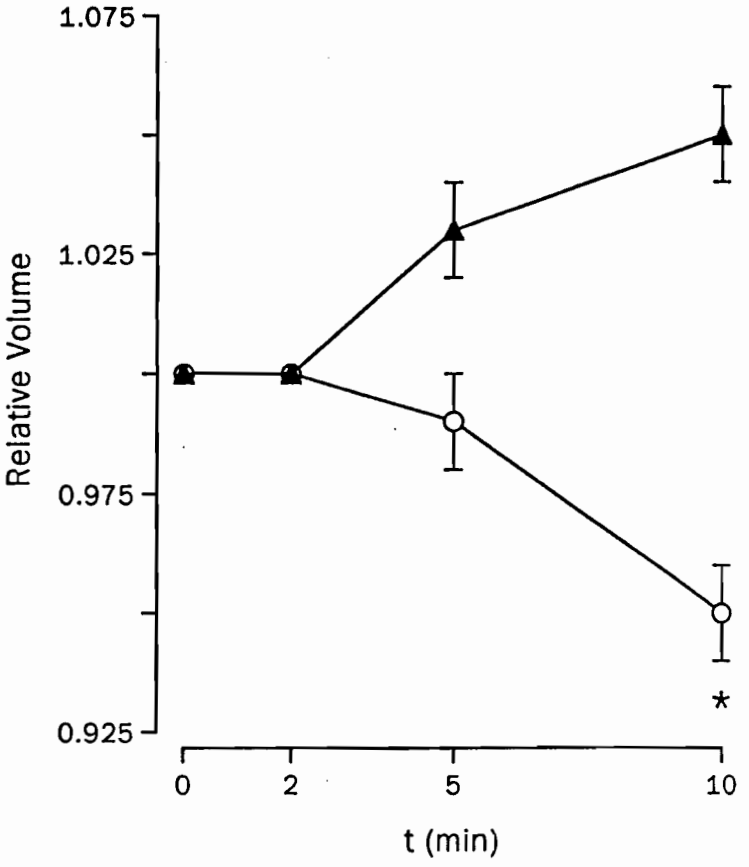
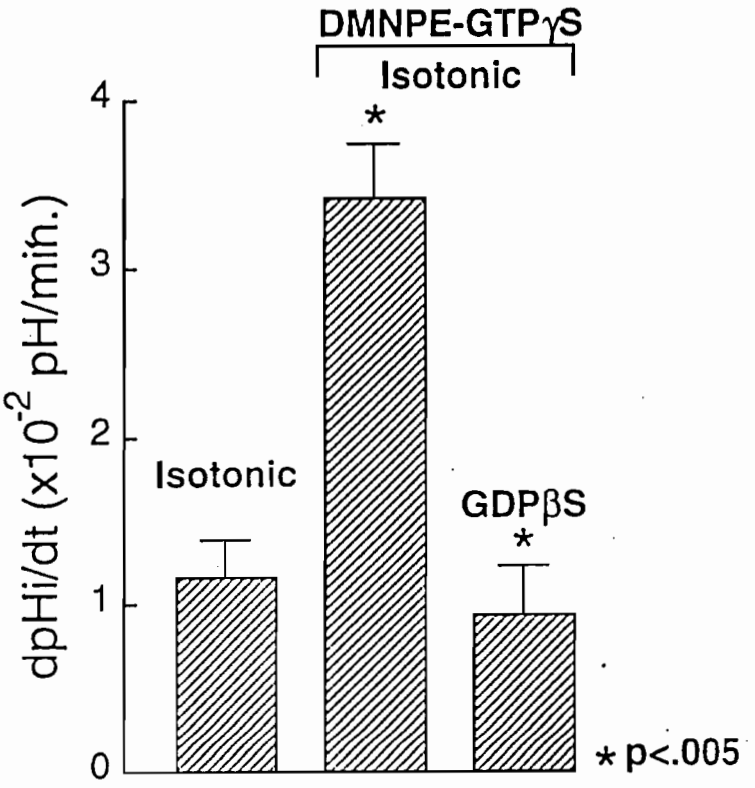

1.075
1.025
0.975
0.925
Relative Volume
0
2
5
10
t (min)
*

Figure Four: Effect of GTPγS and GDPβS on MIA sensitive rate of pH_i recovery from an intracellular acid load. Following incubation in 2.5 mM NH_4Cl for 5 min, rates of MIA sensitive pH_i recovery ($dpH_i/dt \times 10^{-2}$pH units/min) were determined as the difference in isotonic, low Na^+ (25mM) medium containing bafilomycin A_1 (100nM) and Zn^{2+}(100μM) and medium with these inhibitors and 1μM N-5-methyl-isobutyl-amiloride (MIA). Initial starting pH_i was the same (pH_i = 6.90 ± 0.03) for all groups. Data are means ± SE of nine experiments performed in duplicate. *p<0.005, GTPγS vs no additions, GDPβS plus GTPγS.


DMNPE-GTPγS
Isotonic
*
4
3
2
1
0
dpHi/dt (x10-2 pH/min.)
Isotonic
GDPβS
*
* p<.005

Effect of GTPγS, GDPβS on pH_i recovery from acid load.

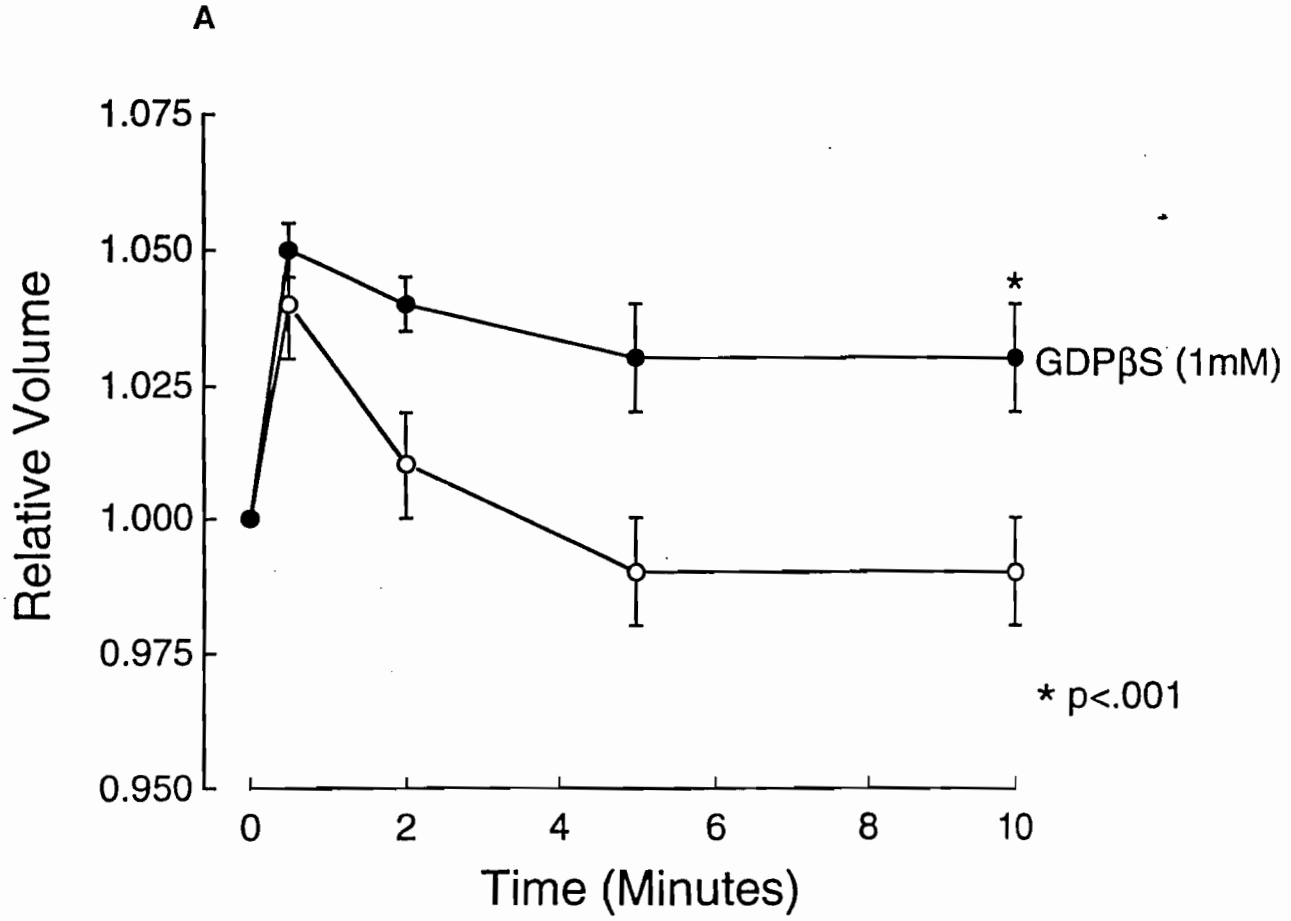
We determined the effect of GTPγS and GDPβS on isotonic NHE activity by measuring the rate of MIA sensitive pH_i recovery (dpH_i/dt) from an acute intracellular acid load induced by an NH_4Cl prepulse (Fig. 4). After resuspension in NH_4Cl free, isotonic medium, the cells were photoactivated. In all experiments, the initial starting pH_i was the same (pH_i=6.90 ± 0.03). In cells loaded with `caged' GTPγS the MIA sensitive rate of $pH_i$ recovery was faster than electroporated but unloaded cells ($dpH_i/dt \times 10^{-2}$: 3.4 ± 0.3 vs 1.2 ± 0.2 pH units $min^{-1}$, $p<0.005$, n=9). This increased rate of $pH_i$ recovery was abolished in cells loaded with 1mM GDPβS and `caged' GTPγS ($dpH_i/dt \times 10^{-2}$: 0.9 ± 0.3 pH units min^{-1}, $p<0.001$, n=9).

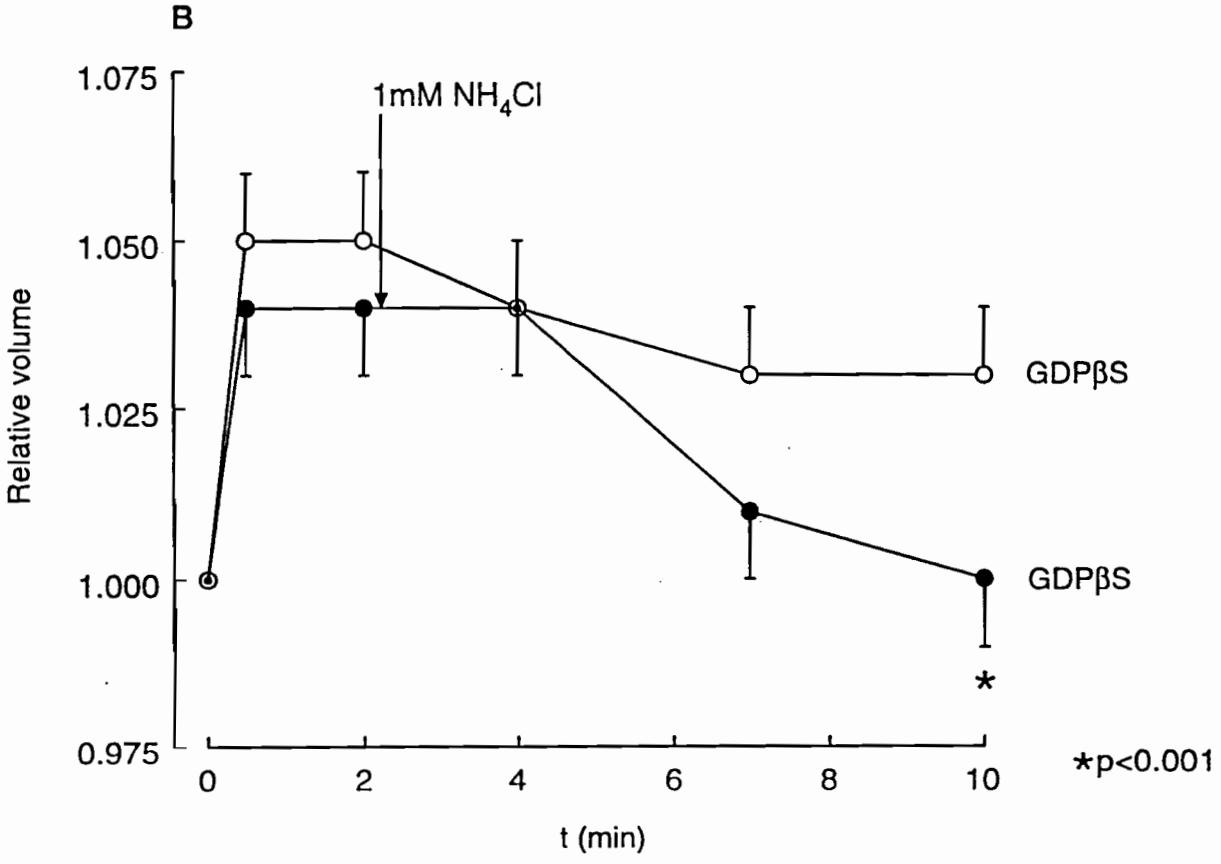
Effect of GDPβS and NH_4Cl induced alkalinization on RVD

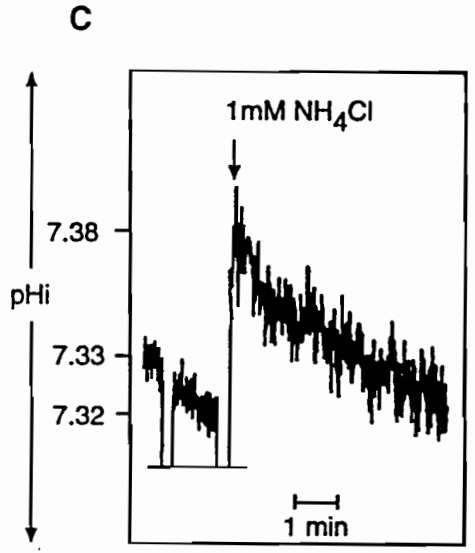
Because loading cells with GDPβS prevented the ΔpH_i stimulated by 5% volume increase, we assessed the role of G proteins in this RVD by measuring volume changes in villus cells loaded with 1 mM GDPβS by electroporation in comparison with controls, electroporated and allowed to reseal (Fig. 5). The RVD after suspension in 5% hypotonic medium in the GDPβS loaded cells was incomplete compared with controls (final relative volume: 1.03 ± .01 vs 0.98 ± 01, $p<0.001$, n=6, Fig 5A). We speculated that, since GDPβS was preventing the pH_i change, causing cell alkalinization should bypass this inhibition and allow RVD to proceed. Addition of 1 mM NH_4Cl to the swollen cells caused RVD in the presence of GDPβS (Fig. 5B). Within 3 min. of the addition of NH_4Cl the cells began to shrink

and the RVD was complete over the next 5 min. (rel. vol. 1.03 ± .01 vs 1.00 ± .01, $p<0.001$).

Figure Five: Effect of GDPβS and NH_4Cl induced alkalinization on RVD. (A) O, Electropermeabilized cells allowed to reseal, 0.95 x isotonic; ●, Permeabilized cells loaded with 1mM GDPβS, 0.95 x isotonic, *$p<0.001$, n=6 (B) ●, NH_4Cl (1mM) added to GDPβS loaded cells, 0.95 x isotonic; O, GDPβS (1mM), 0.95 x isotonic. *$p<0.001$, n=6. (C) pH_i changes of GDPβS loaded cells after 0.95 x isotonic dilution to which NH_4Cl (1mM) was added. Volume measured electronically, expressed relative to isotonic controls.


A
1.075
1.050
1.025
1.000
0.975
0.950
Relative Volume
0
2
4
6
8
10
Time (Minutes)
*
GDPβS (1mM)
* p<.001


B
1.075
1.050
1.025
1.000
0.975
Relative volume
1mM NH4Cl
GDPβS
GDPβS
*
0
2
4
6
8
10
t (min)
*p<0.001

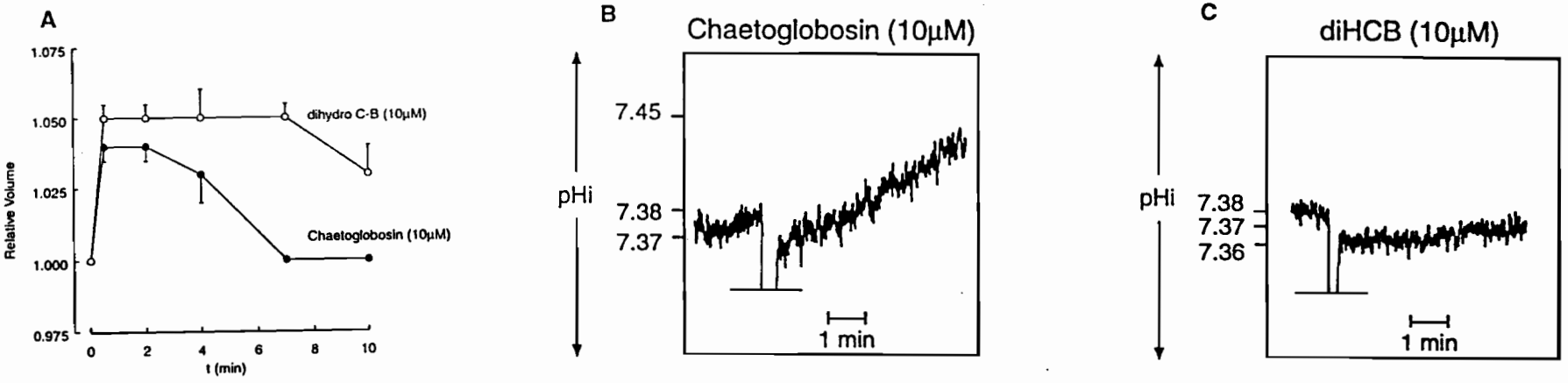

C
1mM NH4Cl
7.38
pHi
7.33
7.32
1 min

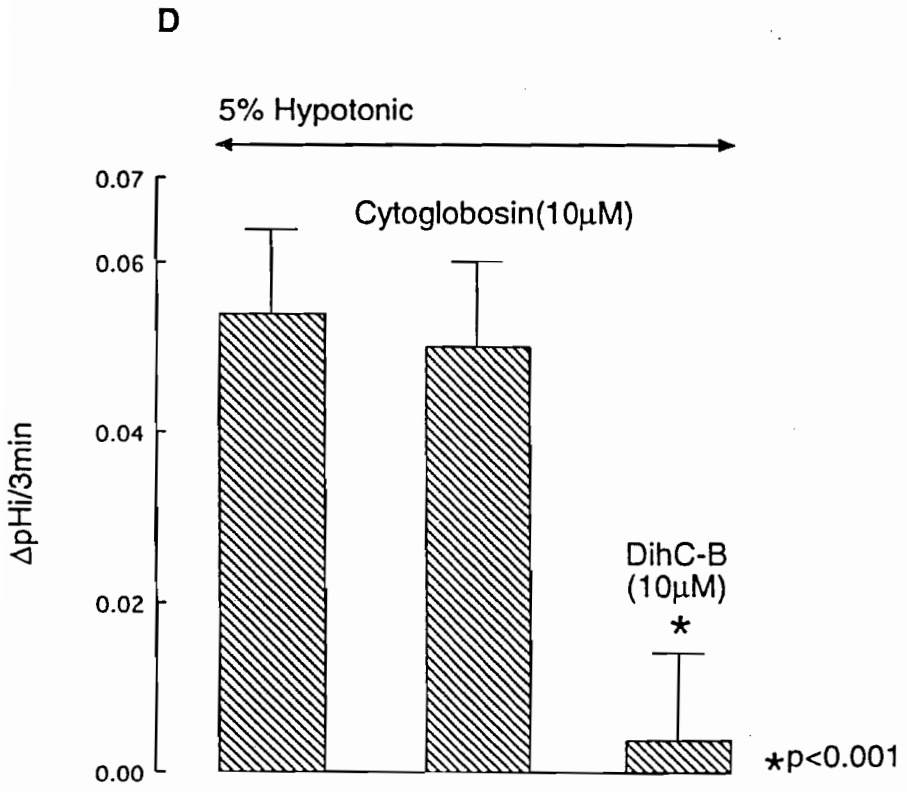
Immediately after the addition of 1 mM NH_4Cl the villus cells alkalinized (Fig. 5C). The extent of this alkalinization (0.050 ± 0.010 pH units) was no different from the alkalinization measured in these cells following 5% hypotonic dilution (0.060 ± .010 pH units, Fig. 1B). These results suggest that the hypotonic activation of NHE is mediated by a G protein but that it is the resultant ΔpH_i which signals the ion channels for subsequent volume regulation.

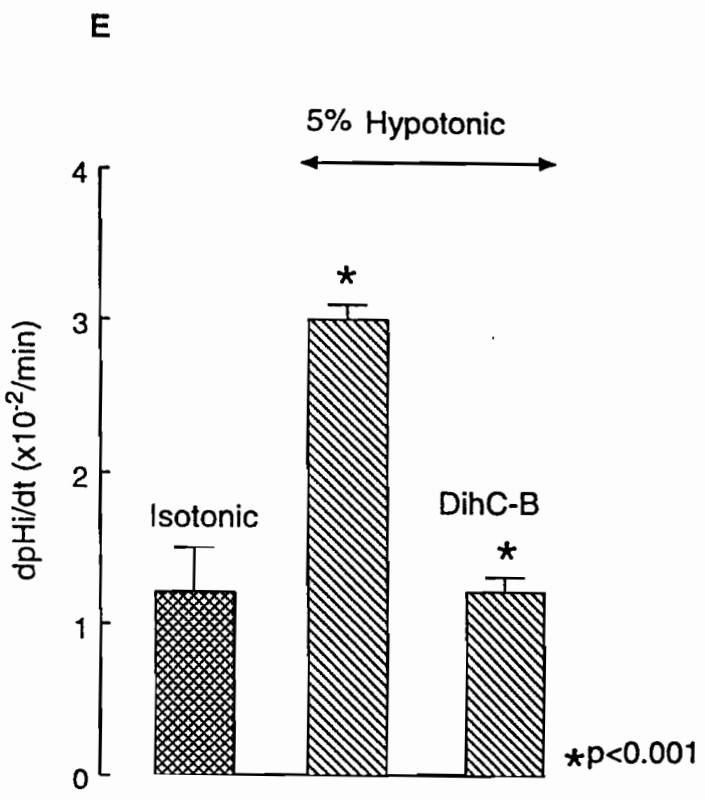
Effect of cytochalasin B on RVD, ΔpH_i and pH_i recovery from an acid load

To determine if the actin-based cytoskeleton contributed to the G protein activation of NHE required for RVD, we used cytochalasins to disrupt F-actin and measured cell volume and pH_i responses to G protein induced RVD (Fig. 6). Dihydrocytochalasin B (10μM, DHCB) prevented RVD ($p<0.001$) in contrast with cells treated with chaetoglobosin C (10μM), a cytochalasin that does not depolymerize actin (Fig. 6A). DHCB (Fig. 5C) also prevented the alkalinization observed after 5% hypotonic dilution compared with cells treated with chaetoglobosin (Fig. 6B). There was no difference in ΔpH_i/3 min after 5% hypotonic dilution in untreated cells compared with cells suspended in medium containing 10μM chaetoglobosin C (ΔpH_i/3 min: 0.050 ± .010). In contrast DHCB (10μM) substantially diminished this ΔpH_i (0.004 ± .010 $p<0.001$, Fig. 6C,D). The initial rate of MIA sensitive pH_i recovery from an acid load after NH_4Cl prepulse was also inhibited by DHCB. Cells were acidified to the same pH_i in all three groups (pH_i= 6.92 ± 0.01). In a medium that was 5% hypotonic the MIA sensitive rate of cell alkalinization was much greater than in an isotonic medium ($dpH_i/dt \times 10^{-2}$: 3.0 ± 0.1

Figure Six: Effect of dihydrocytochalasin B on ΔpH_i, RVD and pH_i recovery from an intracellular acid load. (A) ●, chaetoglobosin (10μM), 0.95 x isotonic; O, Dihydrocytochalasin B (10μM, diHCB), 0.95 x isotonic, n=5. (B,C) ΔpH_i after 0.95 x isotonic dilution after 0.95 x isotonic dilution in presence of either chaetoglobosin or diHCB. Scans corrected for dilutional artifact. (D) ΔpH_i/3 min after 0.95 x isotonic dilution; means ± of five experiments performed in duplicate. *$p<0.001$ vs Chaetoglobosin. (E) MIA-sensitive rate of pH_i recovery in medium that was 0.95 x isotonic according to manipulations described in Methods x $p<0.001$ vs isotonic rate or rate in 0.95 x isotonic medium containing diHC-B (10μM). Means ± SE of five experiments illustrated. Cells acidified to same pH_i (6.92 ± .01) in all three groups.


A
1.075
1.050
1.025
1.000
0.975
Relative Volume
dihydro C·B (10μM)
Chaetoglobosin (10μM)
0
2
4
6
8
10
t (min)
B
Chaetoglobosin (10μM)
pHi
7.45
7.38
7.37
1 min
C
diHCB (10μM)
pHi
7.38
7.37
7.36
1 min


D
5% Hypotonic
ΔpHi/3min
0.07
0.06
0.04
0.02
0.00
Cytoglobosin(10μM)
DihC-B
(10μM)
*
*p<0.001

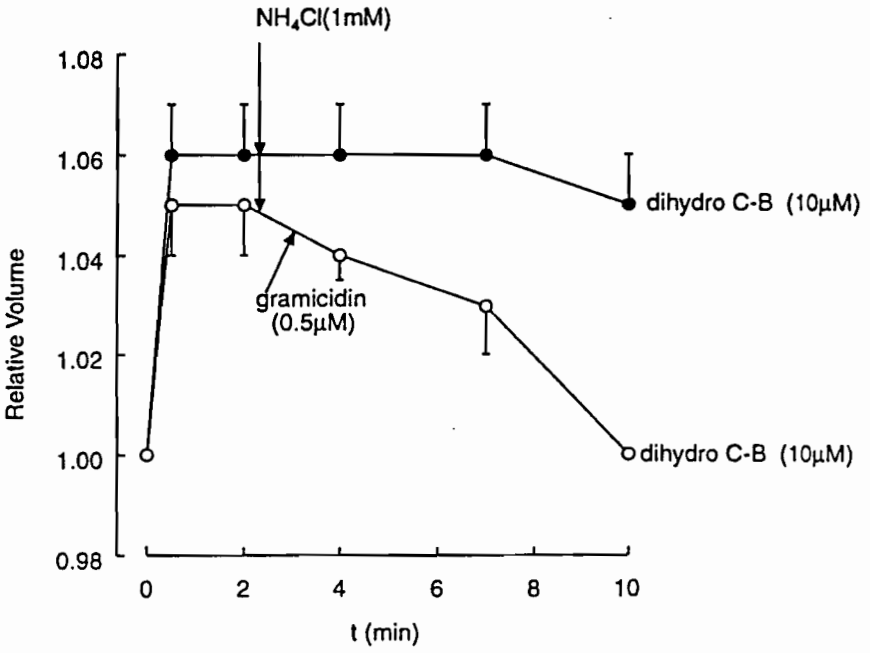

E
5% Hypotonic
dpHi/dt (x10-2/min)
4
3
2
1
0
Isotonic
*
DihC-B
*
*p<0.001

vs 1.2 ± 0.3 pH units min^{-1}, $p<0.001$), but DHCB inhibited this increased rate ($dpH_i/dt \times 10^{-2}$: 1.2 ± 0.2 pH units min^{-1}, $p<.05$, Fig. 6).

Effect of NH_4 or Gramicidin to bypass DHCB inhibition of RVD

To determine if this prevention of RVD by F-actin disruption was because of the failure of the alkalinization of pH_i, or because K^+ or Cl^- channels were directly inhibited, we exposed DHCB treated cells to NH_4Cl alone or to NH_4Cl, and then the cation ionophore, gramicidin (Fig 7). Addition of 1mM NH_4Cl to cells treated with DHCB (Fig. 7) or the Cl^- channel blocker, anthracene-9-carboxylate (A-9C, 300μM) caused an alkalinization (0.050 ± .010 pH units). No RVD was observed after alkalinization in the presence of DHCB (10μM, Fig. 7) or A-9C. Addition of gramicidin to cells treated with A-9C had no effect on volume. However, after alkalinization, gramicidin allowed DHCB treated cells to complete RVD (relative vol. 1.00 ± .01 vs 1.04 ± .001, $p<.05$) suggesting that the K^+ channel, not the Cl^- channel, required polymerized F-actin for activation.

Figure Seven: Effect of NH_4Cl or gramicidin to bypass dihydrocytochalasin B (10μM) inhibition of RVD. ●, diHCB (10μM), 0.95 x isotonic, then 1mM NH_4Cl after 2 min volume measurement. O, diHCB, 0.95 x isotonic, NH_4Cl (1mM) then gramicidin (0.5μM) at 3 min. n=5. Volume measured electronically, expressed relative to isotonic control.


NH4Cl(1mM)
1.08
1.06
1.04
1.02
1.00
0.98
Relative Volume
dihydro C-B (10μM)
gramicidin
(0.5μM)
dihydro C-B (10μM)
0
2
4
6
8
10
t (min)

Effect of Pertussis toxin or staurosporine on RVD

To determine if the G protein activation of NHE in villus cells was pertussis toxin sensitive, we incubated these cells for 0.5 hr with 0.5 μg/mL activated pertussis toxin, then hypotonically diluted them 5%. The toxin treated cells swelled, then exercised complete RVD, exactly like untreated controls (% volume decrease: 5 ± 1 vs 5 ± 1, n=3). The toxin treated cells, however, did not swell following addition of 20 mM L-alanine (rel. vol at 30s 1.05 ± .01 vs 0.95 ± .01, $p<.001$), suggesting that toxin treatment had altered some G protein mediated pathways in these cells. To determine if protein kinase C was involved in the G protein mediated activation of NHE required for RVD we treated the cells with 10 nM of the fungal alkaloid, staurosporine. The staurosporine treated cells were hypotonically diluted 5%. These cells exercised complete RVD, no different than untreated controls (% volume decrease: 5 ± 1 vs 5 ± 1, n=3). In contrast, as previously documented (24), staurosporine blocked RVD in cells swollen because of L-alanine absorption (final rel. vol. 1.04 ± .01 vs 0.95 ± .01, $p<0.001$, n=3).

Discussion

Our results indicate that a G protein mediates the activation of NHE required for RVD following modest, 5 to 7% volume increases of jejunal villus epithelial cells. Several observations support this conclusion. Villus cells loaded with the G protein antagonist, GDPβS, did not generate N-5-alkyl-amiloride sensitive alkalinization after the 5% volume increase, while cells loaded with the G protein agonist, GTPγS manifested an amiloride-sensitive alkalinization in isotonic medium as well as accelerated pH_i recovery from an acid load in isotonic medium; both responses were prevented in cells also loaded with GDPβS. Furthermore, when RVD after the modest volume increase was blocked in cells loaded with the G protein antagonist, causing a comparable alkalinization by NH_4Cl addition allowed RVD to proceed.

Photoactivation of caged GTPγS, loaded into the villus cells by electropermeabilization, resulted in an amiloride sensitive $_{\Delta}pH_i$ that, in the absence of the N-5-alkyl derivative, was prevented by GDPβS in isotonic conditions. As a corollary of the isotonic pH_i experiments, we measured volume changes of these cells after photoactivation and observed cell swelling that was abolished by the amiloride derivative. We interpret these isotonic volume increases as due to G protein activated NHE, with the resultant Na^+ influx osmotically active; the anion component of the volume increase due to volume-activated Cl^- channels which other studies (24) have shown to be activated during isotonic cell swelling after gramicidin permeabilization. Increases in symmetrical cell volume due to NHE activation by intracellular acidification while suspended in isotonic medium is well established (13). Because the MIA sensitive rate of pH_i recovery from an acid load

in 5% hypotonic medium was equivalent (Fig 6B) to photoactivated GTPγS cells in isotonic medium (Fig. 4B), our findings strongly suggest that NHE in these cells is coupled to a G protein. NHE activity in different symmetrical cells has been activated by AlF_5, which mimics the terminal phosphate of GTPγS (34), suggesting the isoform in those cells is coupled to a G protein (18). Hyperosmotic challenge of barnacle muscle fibers results in the activation of NHE whose activity may be regulated by a G protein (10). Our results contrast with others using `caged' GTPγS analogues in canine gut enteroendocrine cells (1). We found that different `caged' GTPγS analogues (DMN, DMNPE) could only be loaded into villus epithelial cells with electropermeabilization, not diffusion (1). For example, incubation of these cells with 100μM GTPγS or 100μM DMNPE-GTPγS stimulated comparable ΔpH_i both of which were prevented by an inhibitor of protein kinase C, staurosporine (10nM) suggesting that the non-hydrolyzable analogue was activating a purinergic receptor. Yet, in electropermeabilized villus cells loaded with GDPβS, the ΔpH_i normally seen after 5% swelling was blocked, as was RVD and the ΔpH_i activated by GTPγS after photolysis in isotonic medium. In a previous study (28) we assessed the identity of the isoform of NHE activated by this modest volume increase by determining the order of potency of non-amiloride inhibitors cimetidine and clonidine; other studies using transfected isoforms of NHE had shown that cimetidine was more potent an inhibitor than clonidine of NHE-1 while for NHE-2 and -3 this order was reversed (38). We found that cimetidine was 6x more potent than clonidine in preventing RVD after the modest swelling, strongly suggestive that NHE-1 was the isoform of NHE activated during the 5% volume increase. Because

these volume and pH_i changes were also blocked by MIA (EC_{50} 220nM) our current experiments suggest that NHE-1 is coupled to a G protein.

Our studies do not identify which Gα subunit is responsible for the activation of NHE in the villus cells. Transient expression of constitutively activated mutants of $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$ and $G\alpha_{13}$ on NHE activity in COS-1 cells have clearly demonstrated that $G\alpha_{12}$ activates NHE through a protein kinase C requiring pathway while $G\alpha_{13}$ does so in a PKC-independent manner (11). Previously, we have reported that PKC inhibitors H-7 and staurosporine have no effect on RVD after 5-7% volume increase to duplicate the volume increase occurring because of L-alanine absorption (24), so it is unlikely that $G\alpha_{12}$ is responsible for the activation of NHE after 5% swelling. Since incubation of the villus cells with pertussis toxin had no effect on RVD after the modest volume increase, our results suggest that the G protein mediating the activation of NHE is pertussis toxin insensitive and PKC-independent.

The MIA inhibition of RVD after modest 5-7% volume increases has previously been shown to be bypassed by alkalinizing villus cells (27,28). The inhibition of RVD seen in cells loaded with GDPβS was bypassed by 1 mM NH_4Cl which caused an alkalinization comparable to that seen when these cells were hypotonically diluted 5% (Fig. 1). We conclude that it is the transient alkalinization of pH_i which determines activation of ion channels for the subsequent osmolyte loss for this volume reduction.

Dihydrocytochalasin B (DHCB), a cytochalasin known to disrupt actin filaments (20,35) prevented the swelling induced ΔpH_i, the pH_i recovery from an

acid load in 5% hypotonic medium, and the subsequent RVD, suggesting intact actin filaments are required for the G protein activation of NHE. Previously, cytochalasin B has been shown to prevent RVD in gallbladder epithelial cells from Necturus (12), fibrosarcoma cells (6), pheochromocytoma cells (7) and Ehrlich ascites cells (8). When NH_4Cl was added to alkalinize the swollen villus cells in the presence of DHCB, no RVD was observed suggesting that either the Cl^- channel or the K^+ channel or both channels required intact microfilaments for activation. After the pH_i alkalinization, gramicidin, a cation ionophore, allowed bypass of RVD suggesting that the K^+ but not the Cl^- channel required intact actin filaments for activation. If it were the Cl^- channel which was responsive to microfilament depolymerization, gramicidin would not have bypassed DHCB inhibition of RVD as the Cl^- channel would not be activated and Cl^- loss would be inhibited. Our results suggest that intact microfilaments, in addition to increases in pH_i, are required to activate the K^+ channel for physiological RVD. This conclusion is in accord with experiments showing a requirement for actin-binding-protein (ABP) in mediating the activation of some K^+ channels (4) where genetic rescue experiments of ABP-deficient cells resulted in osmotic activation of K^+ channels and complete RVD. Since ABP is an actin-filament-cross-linking protein that links actin to various membrane glycoproteins (9,30) these results were consistent with K^+ channels requiring linkage to actin via ABP (4) for osmotic activation. In addition, there is indirect evidence that cytoskeletal elements will modulate the activity of NHE. The adherence of symmetrical cells promotes activation of NHE-1 which is mediated by integrin $\alpha_5\beta_1$ (32). Immunofluorescence experiments have shown in adherent

symmetrical cells that NHE-1 accumulates in sites which are also sites of accumulation of vinculin, tailin and F-actin (15). Some integrins will serve as anchors for cytoskeletal structures composed of tailin and actin (32). While no direct evidence links NHE-1 with a cytoskeletal element, in renal epithelia the Na^+/K^+ ATPase is linked to actin by ankyrin and spectrin (29). Furthermore, Na^+ channels in A6 epithelial cells will increase their open probability by cytochalasin D treatment and immunochemical evidence has shown these channels localized with actin, spectrin and ankyrin in the microvilli of these cells (33). Because DHCB prevented the swelling induced $_{\Delta}pH_i$, our results suggest that an intact actin cytoskeleton is necessary to transduce the G protein-mediated activation of NHE resulting from the modest volume increases. Actin microfilaments could be linked to a putative receptor/volume sensor which is coupled to a G protein; alternatively, some microfilaments might be directly coupled to a trimeric G protein or indirectly linked to NHE. Our experiments do not allow us to discriminate between these models, but the latter is unlikely because acid-loaded enterocytes still manifested MIA-sensitive pH_i recovery in the presence of DHCB (Fig 5C). Therefore, we speculate that if a G protein complex were directly coupled to actin microfilaments, mechanical stretching associated with a physiological volume increase of 5 to 7% might directly activate the G-protein via these microfilaments, in a manner analgous to the conformational change of a receptor caused by agonist binding leading to G protein activation (16,17). In these epithelial cells, the volume sensor would be a microfilament-linked G protein. There is evidence that G proteins are coupled to some cytoskeletal elements. In symmetrical cells β subunits of G proteins co-

fractionate with cytoskeletal actin with differential detergent extraction (5), and several Gα subunits will bind specifically to tubulin (37). Nevertheless, it is clear from our experiments that disrupting the actin cytoskeleton with a cytochalasin derivative prevents the G protein mediated activation of NHE which is required for complete RVD.

In summary, cell swelling of a degree to be expected during Na^+-solute cotransport (5-7%) results in an amiloride sensitive alkalinization of pH_i in villus enterocytes. Preventing this alkalinization prevents RVD. GDPβS loaded cells do not alkalinize with 5% swelling or exercise RVD. In isotonic medium, GTPγS will activate methyl-isobutyl-amiloride sensitive pH_i changes which are prevented by GDPβS. The MIA sensitive rate of pH_i recovery from an acid load is increased in 5% hypotonic medium compared with isotonic medium but equivalent to GTPγS treated cells in isotonic medium. Treatment of cells with dihydrocytochalasin B blocks the pH_i alkalinization after 5% volume increases and the subsequent RVD suggesting intact microfilaments are required to activate NHE. In cells loaded with GDPβS, addition of NH_4Cl causes pH_i alkalinization which results in complete RVD. We conclude that the activation of NHE required for RVD following modest, but probably physiological, volume increases in these cells is mediated by a G protein.

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A G-protein mediates the activation of Na^+/H^+ exchange (NHE) when villus cells swell a modest (5 to 7%) amount. The alkalinization of pH_i resulting from the activated NHE is an absolute requirement for the subsequent volume recovery (RVD). This RVD is due to K^+ and Cl^- efflux from volume-activated channels. To determine the relationship between pH_i changes and the K^+ channels activated for RVD after modest volume increases, experiments were designed to answer the following questions: Are charybdotoxin-sensitive, Ca^{2+}-activated K^+ channels responsible for K^+ loss after modest (5 to 7%) volume increases compared with greater (12 to 15%) volume increases? Is ^{86}Rb efflux stimulated by 7% volume increases influenced by inhibitors of NHE? After greater volume increases does causing an alkalinization of pH_i activate charybdotoxin-sensitive K^+ channels? To answer these questions we assessed the rate of ^{86}Rb efflux from cells in response to hypotonic dilution and measured pH_i and volume responses of cells alkalinized by either NH_4Cl addition or nigericin addition in alkaline (pH_o=7.56) medium.

Chapter Four

Increases in intracellular pH are required for K^+ channel activation after modest `physiological' swelling in villus epithelial cells

Summary

We studied the relationship between changes in intracellular pH (pH_i) and charybdotoxin sensitive (CTX) maxi-K^+ channels after modest `physiological' amounts of cell swelling in guinea pig jejunal villus enterocytes. Cell volume was assessed using electronic cell sizing and pH_i was measured by fluorescence spectroscopy of villus cells in suspension loaded with 2,7, biscarboxyethyl-5-6-carboxyfluorescein. Following a 0.93 x isotonic dilution, the villus cells swelled to the same size they reach because of D-glucose or L-alanine absorption. The subsequent Regulatory Volume Decrease (RVD) was prevented by CTX. Greater volume increases caused by 0.80 x isotonic dilution were followed by RVD unaffected by CTX. After 0.93 x isotonic dilution, the pH_i alkalinized: N-5-methyl-isobutyl amiloride (MIA) prevented this ΔpH_i and the subsequent RVD. When volume regulation was blocked by MIA, NH_4Cl addition caused an alkalinization of pH_i which resulted in complete RVD. CTX blocked the NH_4Cl bypass of the MIA inhibited RVD. The rate of ^{86}Rb efflux was increased by 0.93 x isotonic dilution. This increased rate was inhibited an equivalent amount by CTX, MIA or Na^+-free medium. After a 0.50 x isotonic dilution in K^+ rich medium, alkalinizing the pH_i, either by NH_4Cl addition or adding nigericin when medium pH_o=7.56, resulted in volume increases that were CTX-sensitive. A membrane permeant inhibitor of protein kinase A, Rp-cAMPS, prevented 8-Br-cAMP induced volume changes, but had no effect on RVD after 0.93 x isotonic dilution. We conclude that the alkalinization of pH_i observed with `physiological' volume increase is a critical determinant of the activation of CTX-sensitive maxi-K^+ channels required for RVD.

Introduction

In jejunal villus epithelial cells it is the extent of cell swelling that determines the signal transduction pathway controlling the K^+ and Cl^- channels responsible for the Regulatory Volume Decrease (RVD). High conductance Ca^{2+}-activated (maxi-K^+) K^+ channels which are sensitive to charybdotoxin (CTX) are essential for RVD after the volume increases of 5 to 7% occurring because of Na^+-solute absorption, but not for RVD after greater volume increases (15%) occurring after `standard', 0.70 x isotonic dilution (MacLeod et al., 1992; MacLeod & Hamilton, 1991A,B). Because our preliminary studies (MacLeod et al., 1994A) have recently found a distinctive intracellular pH (pH_i) change which accompanies such modest, 5 to 7% volume increases, the current experiments focus on the relationship between intracellular alkalinization and K^+ channel activation after volume increases caused by hypotonic dilution of suspended jejunal villus cells. We compared the response of volume increases of 5 to 7% which replicate the response to Na^+-glucose absorption with a more substantial 12 to 15% volume increase (0.8 to 0.7 x isotonic) routinely used in volume regulatory studies (Foskett, 1994).

Materials and Methods

Villus isolation and volume determinations

Villus cells were isolated from segments of adult male (200-300g) guinea pig jejunum by mechanical vibration as previously described (MacLeod & Hamilton, 1990). Isolated cells were resuspended at 0.8-1.5 x 10^6 cells/mL in RPM1-1640 median (without HCO_3) containing bovine serum albumin (type V) at 1 mg/mL and 20mM Na Hepes, pH 7.3 at 37°C. Viability, assessed by trypan blue exclusion was 85%, 3 hr after suspension in medium. Cell volume was measured using a Coulter Counter (Zm) with an attached Channelyzer (C-256) as previously described (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, 1991B). Relative cell volume was expressed as the ratio of cell volume under experimental conditions to volume under basal conditions in an isotonic medium immediately before challenge.

pH_i measurements

pH_i was determined fluorometrically using BCECF. We loaded villus cell suspensions (1x 10^6 cells/mL in Hepes RPMI) with BCECF by incubation with the parent acetoxymethylester (3.7μM) for 15 min at 37°C. After washing, and resuspension, 0.5 to 0.8 x 10^6 cells were used for fluorescence determination in 2 mL of the indicated medium 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 (Thomas et al, 1979)

and a quench correction factor as described (Grinstein et al., 1989). The resting pH_i of these cells in Hepes buffered RPMI-1640 medium (nominally HCO_3-free) was 7.39 ± 0.04, n=45.

^{86}Rb efflux measurements

We measured ^{86}Rb efflux from the villus cells using a technique we have previously described (MacLeod et al., 1992C). Villus cells ($2x10^6$ cells/mL) were loaded in RPMI medium with ^{86}Rb (10μCi/mL) for 45 min. Cell suspensions were diluted fivefold with ^{86}Rb-free RPMI, centrifuged, and resuspended in Na^+ medium, pH 7.3, at $3\text{-}5x10^6$ cells/mL. One mL of this suspension was removed and diluted isotonically 7% with Na^+ medium; then 500μL of the cell suspension was 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 100μL of a 3:2 (vol/vol) di-n-butyl- phthalate: di-n-nonylphthalate oil mixture and centrifuged at 1300g for 20 s 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 suspension, aliquots of cell suspension were added to an ice-cold stop solution that was 0.93 x 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 lysed in 0.5 ml of 1% sodium dodecylsulfate. Radioactivity associated with the cell pellet was assessed with liquid scintillation counting. Results were expressed as the fraction of isotope remaining over time compared with the 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, is osmotically replaced by KCl. Na^+-free medium was prepared by isosmotically replacing NaCl with the chloride salt of N-methyl-D-glucamine ($NMDG^+$). pH_i measurements were made in Na^+ medium, and where indicated, K^+ rich medium.

Reagents

The acetoxymethyl ester of 2', 7' bis (carboxyethyl) 5-(6)-carboxyfluorescein (BCECF) was 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 are reported as means ± SE of five to seven experiments performed in duplicate. Differences in means were determined using Student's t test.

Results

Effect of charybdotoxin (CTX) on RVD after 0.93 x isotonic or 0.80 x isotonic dilutions

We suspended villus epithelial cells in hypotonic medium, 0.93 x 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 1A). 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 x isotonic, causing a greater volume increase, underwent RVD which was not affected by 50 nM CTX (Fig. 1B). 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.

Effect of CTX on changes in intracellular pH (pH_i) after 7% hypotonic dilution

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 x isotonic medium, the cells alkalinized (Fig 2A). 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. 2B) but CTX (50 nM) had no effect on the alkalinization (ΔpH_i/3 min: 0.080 ± 0.10 vs 0.070 ± 0.010 pH units; Fig 2C). Subsequent RVD was also prevented by the amiloride derivative (Fig. 2D); the final relative volume of cells was larger in the presence of 1 μM MIA compared

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

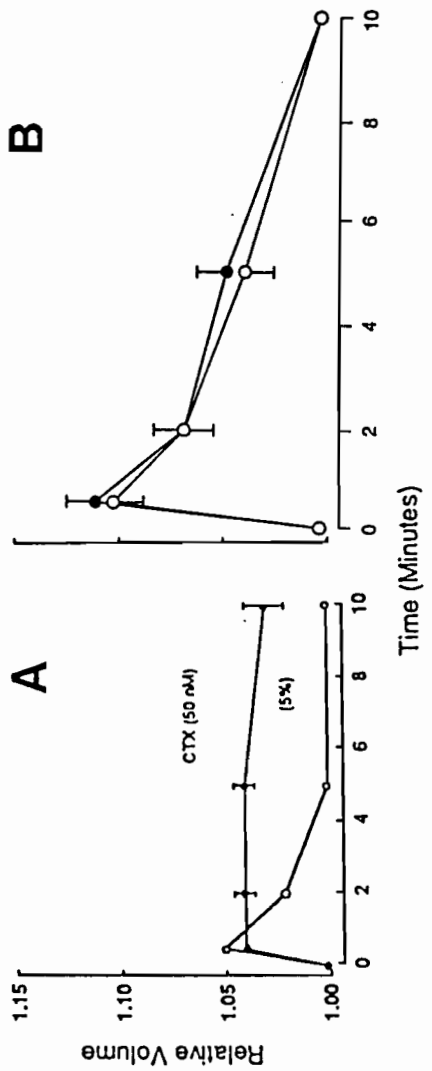
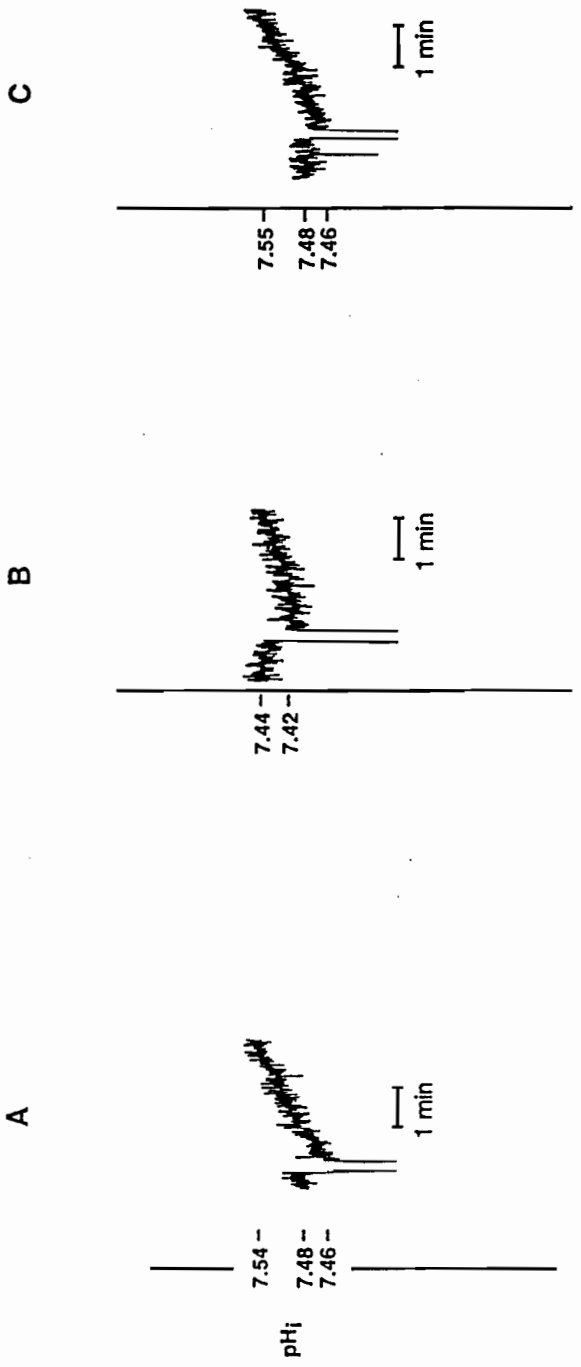
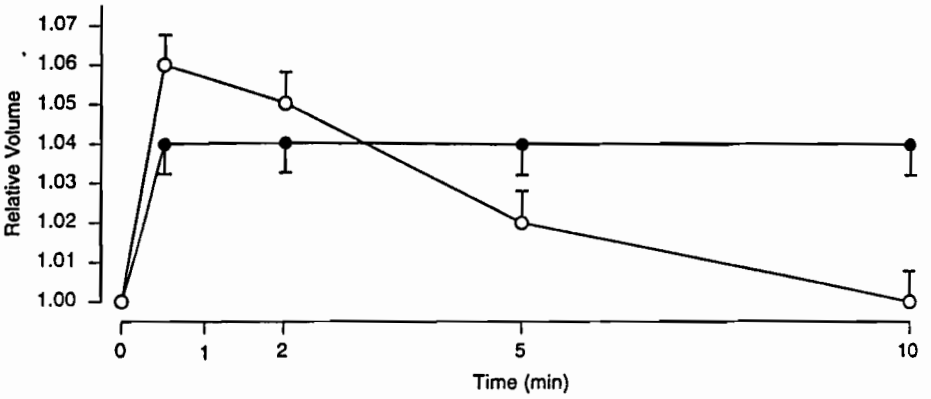

A
B
CTX (50 nM)
(5%)
Relative Volume
1.15
1.10
1.05
1.00
0
2
4
6
8
10
Time (Minutes)

Figure 2: Changes in pH_i and volume after 0.93 x isotonic dilution. (A) Alkalinization after 7% hypotonic dilution. (B) Effect of 1 μM N-5-methyl-isobutyl amiloride (MIA). (C) Effect of CTX (50nM) on ΔpH_i after 7% hypotonic dilution. (D) Effect of MIA (1μM) on RVD after 7% hypotonic dilution. 0, 0.93 x isotonic; ●, MIA (1 μM) + 0.93 x isotonic, n=5. Volume measured electronically, expressed relative to isotonic control.


A
B
C
1 min
1 min
1 min
7.54
7.48
7.46
pHi
7.44
7.42
7.55
7.48
7.46

D


Relative Volume
1.07
1.06
1.05
1.04
1.03
1.02
1.01
1.00
0
1
2
5
10
Time (min)

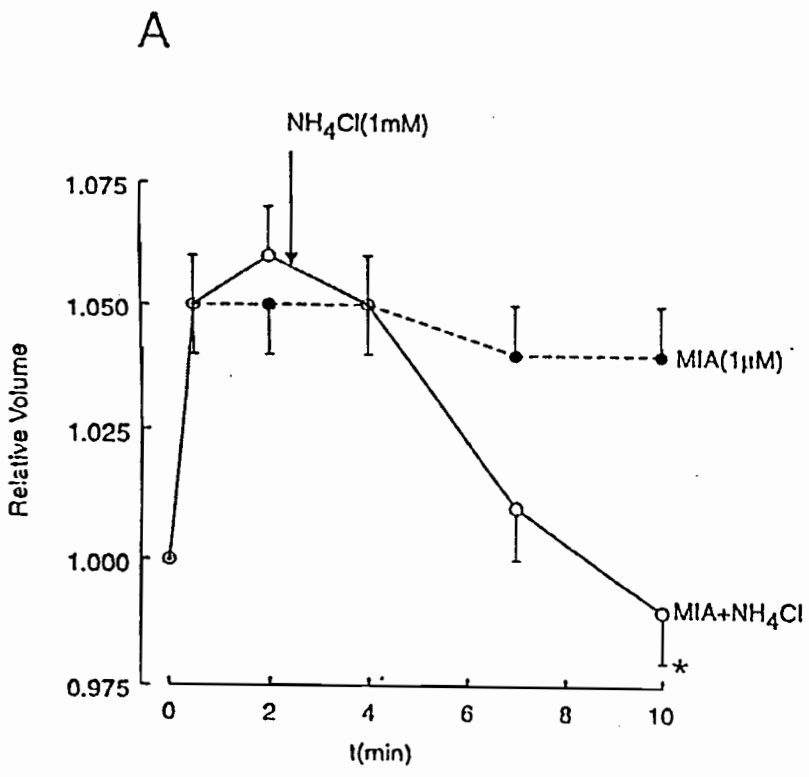
with untreated controls (1.04 ± .01 vs 1.00 ± .01, $p<0.001$). These results suggested that the amiloride-sensitive alkalinization seen after modest volume increase was required to activate the K^+ or Cl^- channels necessary for RVD.

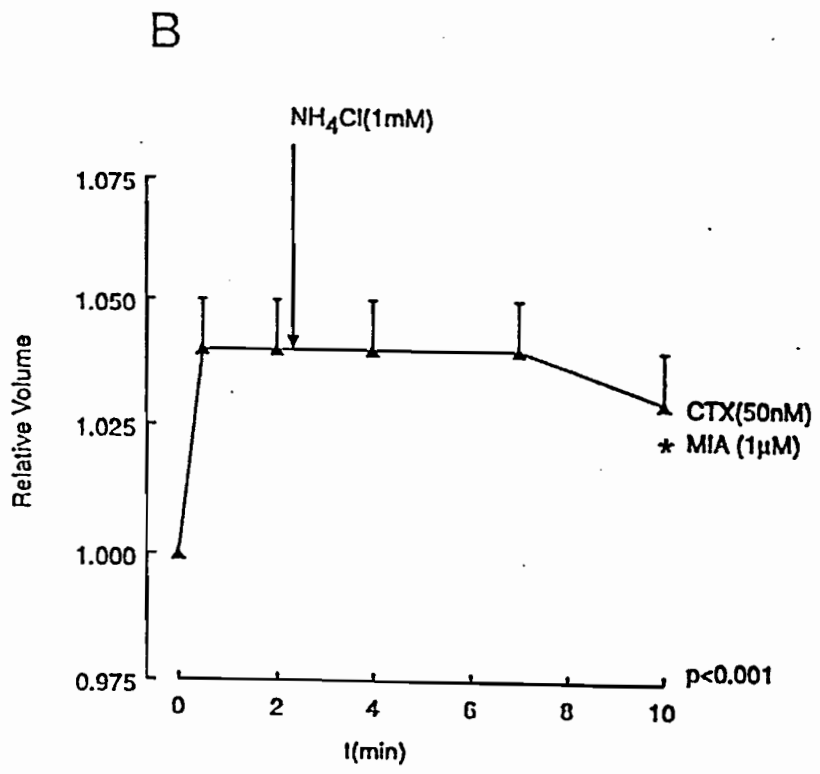
This prevention of RVD by 1 μM MIA was bypassed by 1 mM NH_4Cl (Fig. 3C) which caused an alkalinization of 0.080 ± 0.010 pH units and resulted in a complete volume recovery (Fig. 3A). The final relative volume of cells treated with NH_4Cl and MIA was less than cells treated with MIA alone (0.99 ± .01 vs 1.04 ± .01,$p<0.001$). In contrast, the addition of CTX (50nM) to cells hypotonically diluted 7% in the presence of MIA and 1 mM NH_4Cl, prevented RVD (Fig. 3B). The final relative volume of CTX treated cells was larger than those treated with MIA and NH_4Cl but no CTX (1.03 ± .01 vs 0.99 ± .01, $p<0.001$). These data suggest 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 x 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, $p<0.02$, $n=5$).

Figure 3: Effect of CTX on NH_4Cl bypass of methyl-isobutyl-amiloride inhibition of RVD. (A) ●, 0.93 x isotonic, 1 μM MIA; ○, 0.95 x isotonic, 1 μM MIA, 1 mM NH_4Cl. (B) ▲, CTX (50nM), MIA (1 μM), NH_4Cl (1mM), 0.93 x isotonic. (C) Representative pH_i tracing of addition of 1 mM NH_4Cl to cells after 0.93 x isotonic dilution in the presence of 1 μM MIA. * $p<0.001$. Volume measured electronically, expressed relative to isotonic control.


A
NH4Cl(1mM)
1.075
1.050
1.025
1.000
0.975
Relative Volume
MIA(1μM)
MIA+NH4Cl
*
0
2
4
6
8
10
t(min)


B
NH4Cl(1mM)
1.075
1.050
1.025
1.000
0.975
Relative Volume
CTX(50nM)
* MIA (1μM)
p<0.001
0
2
4
6
8
10
t(min)

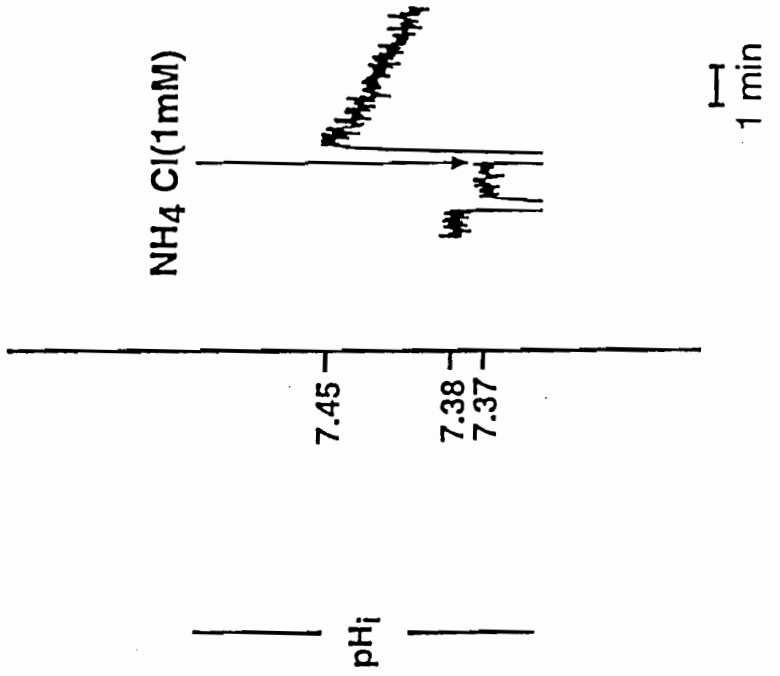
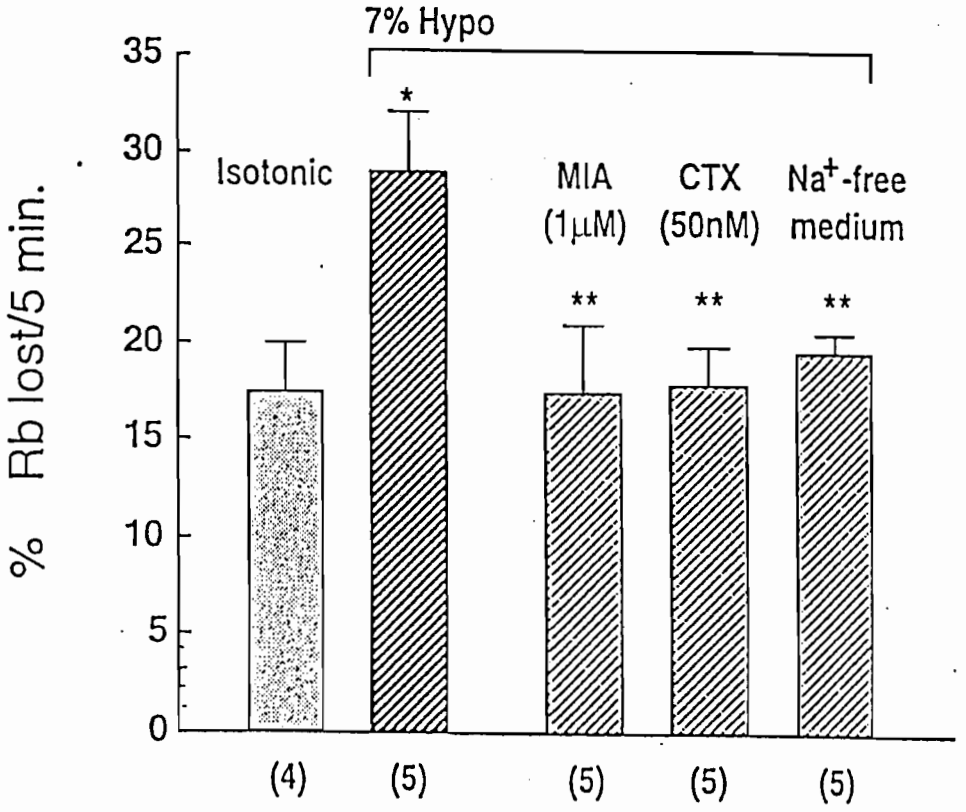

NH4 Cl(1mM)
1 min
7.45
7.38
7.37
pHi

Figure 4: Effect of CTX, N-methyl-isobutyl-amiloride (MIA), Na^+-free medium on ^{86}Rb efflux after 7% hypotonic dilution. MIA (1µM); CTX (50nM); Na^+ free medium prepared by isotonically 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.

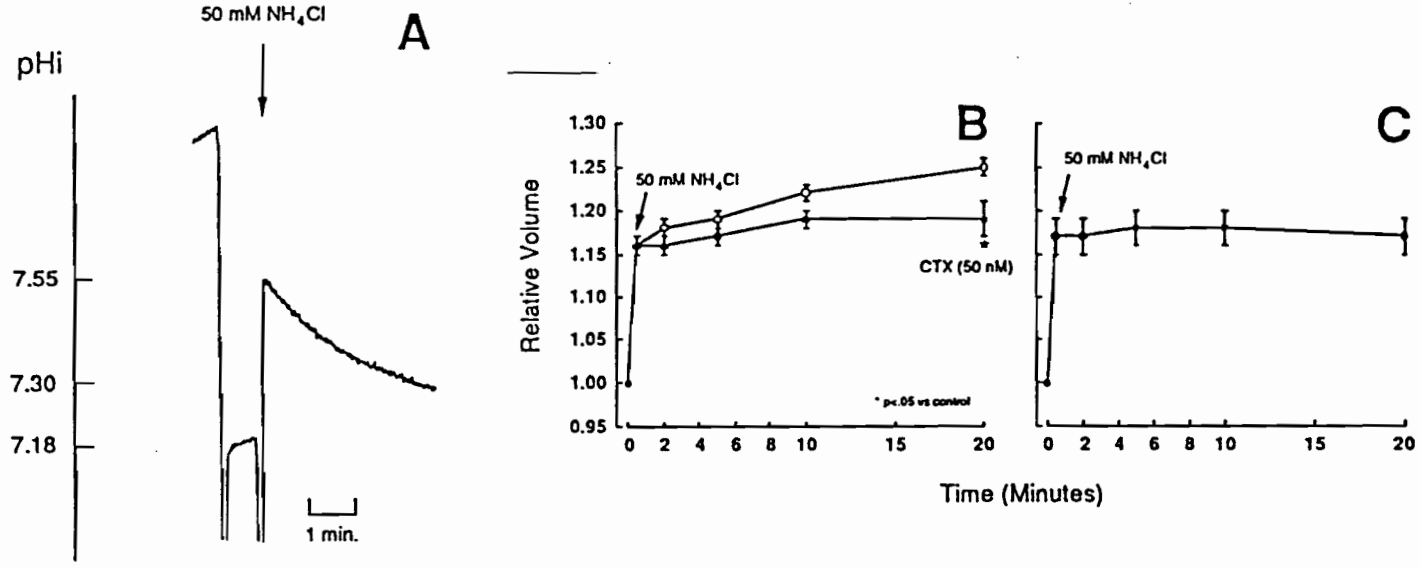

7% Hypo
35
30
25
20
15
10
5
0
% Rb lost/5 min.
Isotonic
MIA (1μM)
CTX (50nM)
Na+-free medium
*
**
**
**
(4)
(5)
(5)
(5)
(5)
*p<.025 vs Isotonic
**p<.020 vs Hypo

There were no differences in rates of ^{86}Rb loss when the effect of CTX was compared with MIA or Na^+-free conditions. These results suggest that it was the CTX-sensitive K^+ channel which was responsive to the 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 x isotonic dilution.

Alkalinizing pH_i activates CTX-sensitive secondary cell swelling

To determine whether activation of CTX-sensitive K^+ channels required an alkalinization of pH_i, we first generated volume increases where CTX-insensitive K channels were activated (volume increases > 15% after 0.5 x isotonic dilution), then made the pH_i more alkaline and assessed whether subsequent volume changes were CTX-sensitive. The rationale of this approach was that the direction and extent of volume regulation of these cells has 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, 1991B). After suspending cells in 0.5 x isotonic K^+ rich medium, the addition of 50 mM NH_4Cl (Fig. 5A) resulted in an alkalinization of ≈0.39 pH units. This manoeuvre resulted in a sustained secondary cell swelling (Fig. 5B) (rel. vol.: 1.25 ± .01 vs 1.16 ± .01, $p<0.05$), which was completely prevented by CTX (50nM) (final rel. vol. 1.19 ± 0.02, $p<0.05$).

Figure 5: 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 x isotonic. Tracing not corrected for dilutional artifact. (B) Cell volume changes in response to these manipulations. ●, CTX (50nM), 0.5 x isotonic, 50mM NH_4Cl. ○, 0.5 x isotonic, 50mM NH_4Cl. (C) ●, 0.5 x isotonic, 50mM NH_4Cl in Ca^{2+} free (150 µM EGTA) K^+ rich medium. n=5. *$p<0.05$ vs control. Volume measured electronically, expressed relative to isotonic control.


50 mM NH4Cl
A
pHi
7.55
7.30
7.18
1 min.
B
C
50 mM NH4Cl
CTX (50 nM)
* p<.05 vs control
Relative Volume
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0.95
0 2 4 6 8 10 15 20
Time (Minutes)

In nominally Ca^{2+} free medium, containing 150 μM EGTA, there was no secondary swelling response to alkalinization (Fig. 5C) (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. In additional experiments, we duplicated the extent of alkalinization observed with modest 5% volume increase by adding 1 mM NH_4Cl (final) to cells immediately after a dilution of 0.5 x isotonic in the K^+ rich medium. This manoeuvre caused an alkalinization of 0.070 ± 0.010 pH units, and a transient cell swelling occurred. The relative volume of these cells was greater at 5 min compared with 30s (rel. vol: 1.59 ± .02 vs 1.53 ± .02, $p<0.05$, n=8). CTX prevented this transient cell swelling (rel. vol. 1.53 ± .01, $p<.05$, n=8). To other cells, we controlled for the increased osmolarity by adding 1 mM NaCl. There was no secondary swelling in these cells, (rel. vol. 1.56 ± .02 vs 1.54 ± .02, n=8).

Nigericin induced alkalinization activates CTX-sensitive swelling

As an alternative to NH_4Cl addition, we used the K^+/H^+ exchanger, nigericin, to alkalinize the pH_i of cells that had swollen > 15% after substantial hypotonic dilution (Fig. 6). In K^+ rich medium, pH_o=7.56, villus cells were diluted 0.5 x isotonic, and nigericin (10μM) was immediately added to cause secondary swelling (rel. vol. 1.53 ± .02 vs 1.45 ± .01, $p<0.005$, n=6). CTX prevented the transient secondary swelling (rel. vol. 1.49 ± .01 vs 1.45 ± .02, n=6). In contrast, at pH_o = 7.30, the addition of nigericin did not cause swelling (rel. vol. 1.44 ± .01 vs 1.43 ± .01, n=6). At pH_o = 7.30, CTX had no effect on cell volume after the addition of

nigericin (rel. vol. 1.44 ± .01 vs 1.43 ± .01, n=6). Together the results illustrated in Figure 5 and 6 suggest that while RVD after volume increases ≥ 15% are insensitive to CTX, if the pH_i of these cells is made more alkaline, the volume response becomes CTX-sensitive.

Effect of PKA inhibition on 0.93 x isotonic RVD

Because some CTX-sensitive maxi K^+ channels may be activated by phosphorylation via protein kinase A (Reinhart, et al., 1991) we determined whether an inhibitor of PKA might alter CTX-sensitive RVD (Fig. 7). The addition of 8-Br-cAMP (0.5 mM) to the villus cells in isotonic medium resulted in a slight volume reduction (Fig. 7A). In the presence of the PKA antagonist, Rp-cAMPS (10μM), 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 modest volume increase (Fig. 7B). These results suggest that PKA was not responsible for the activation of the CTX-sensitive K^+ channel required for RVD.

Figure 6: Nigericin addition in alkaline (pH_o=7.56) K^+ rich medium causes CTX-sensitive secondary volume changes. Villus cells suspended in K^+ rich, alkaline (pH_o 7.56) medium, diluted 0.5 x isotonic and nigericin (10 μM) added. ●, pH_o 7.56; ○, pH_o 7.56, CTX (50nM); Δ, pH_o 7.3. *$p<0.005$ vs CTX. n=6. At pH_o 7.3, CTX no effect on volume changes. Volume measured electronically, expressed relative to isotonic control.

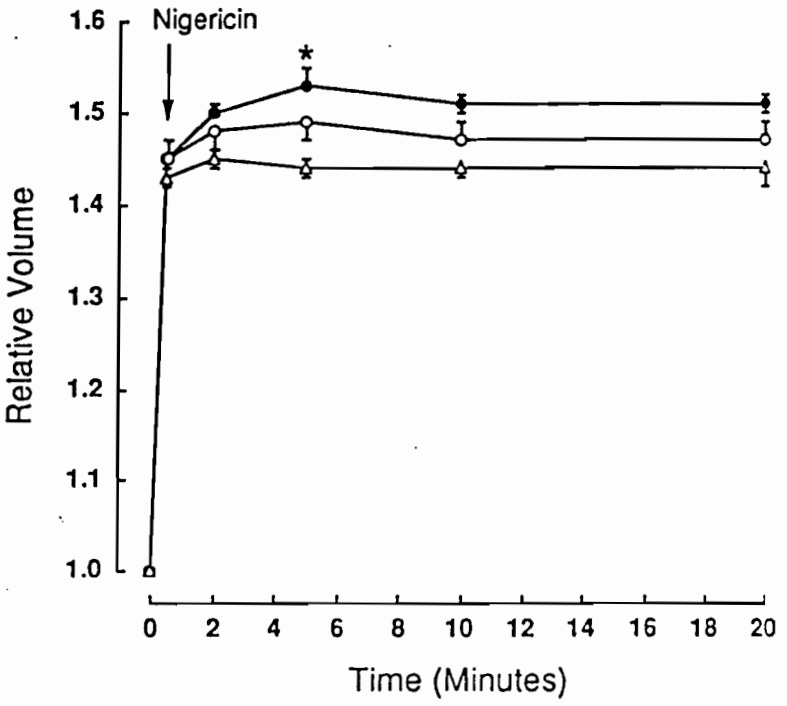
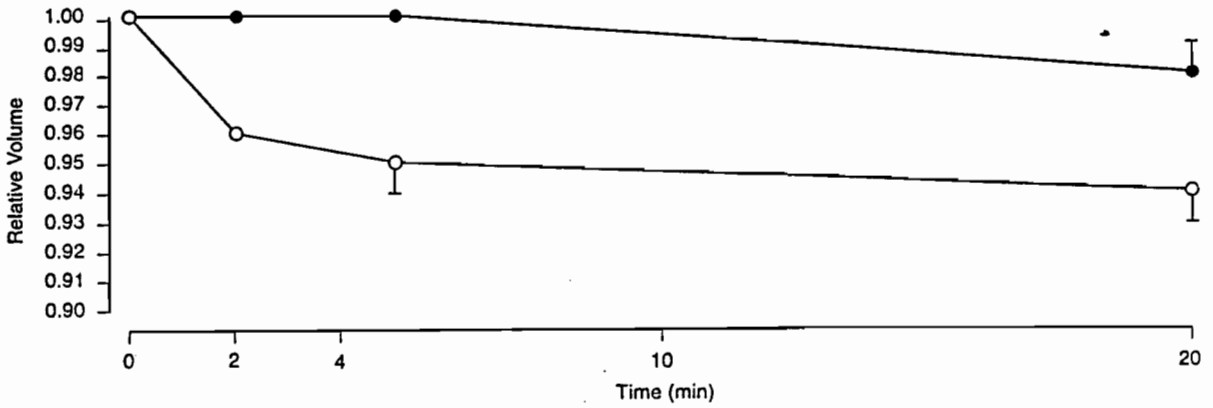
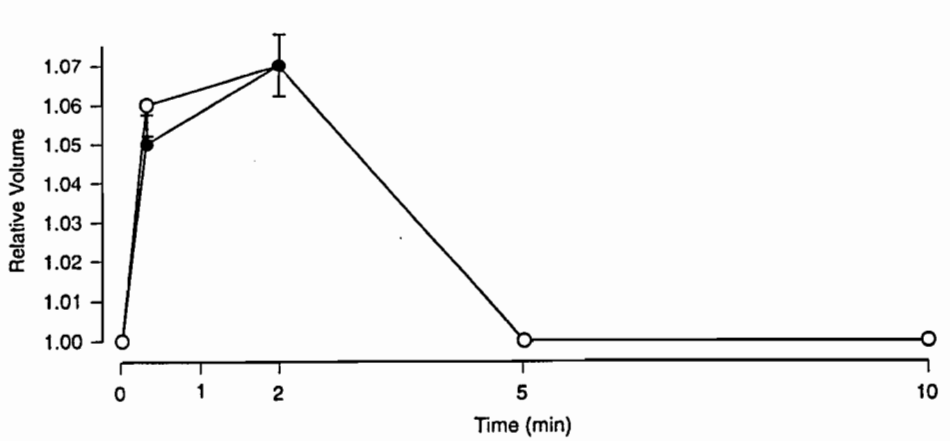

Nigericin
*
1.6
1.5
1.4
1.3
1.2
1.1
1.0
Relative Volume
0
2
4
6
8
10
12
14
16
18
20
Time (Minutes)

Figure 7: Effect of Rp cAMPS on 8-Br-cAMP induced volume changes and RVD after 0.93 x isotonic dilution. (A) ○, 8-Br-cAMP (0.5 mM); ●, Rp cAMPS (10 μM) + 8-Br-cAMP. (B) ○, 0.93 x isotonic; ●, 0.93 x isotonic, Rp cAMPS (10 μM) n=4. Volume measured electronically, expressed relative to isotonic control.

A


Relative Volume
1.00
0.99
0.98
0.97
0.96
0.95
0.94
0.93
0.92
0.91
0.90
0
2
4
10
20
Time (min)

B


Relative Volume
1.07
1.06
1.05
1.04
1.03
1.02
1.01
1.00
0
1
2
5
10
Time (min)

Discussion

The current studies further characterize the distinctive volume regulatory mechanisms activated by a modest 5-7% cell swelling occurring after exposure to a slightly hypotonic (0.93 -0.95 x isotonic) medium constituted to duplicate the extent of swelling when the same cells absorb D-glucose or L-alanine (MacLeod & Hamilton, 1991; MacLeod et al., 1992A,B). The charybdotoxin (CTX) sensitive K^+ channel activated by this modest but probably `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. These findings differ from those observed in the same cells after larger (12 to 25%) volume increases, where the Regulatory Volume Decrease (RVD), which is CTX-insensitive, is associated with cell acidification not alkalinization (MacLeod et al., 1994A). In the current studies, alkalinization of cells after volume increases $\geq$ 12% resulted in volume changes that were CTX-sensitive. Our results indicate that the increase in pH_i after modest `physiological' cell swelling of villus epithelial cells is a critical determinant of the activation of the CTX-sensitive K^+ channel required for 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, block with high affinity in several epithelia (Clarke et al., 1993; Cornejo et al., 1984; Garcia et al., 1995; Tanc et al., 1993; Lu et al., 1993). Other maxi K^+ channels are insensitive to the toxin (Reinhart et al., 1991). Single-channel analysis of maxi-K^+ channels from the basolateral membrane of rabbit colonocytes (Klaerke, D.A. et al., 1993) or

rat skeletal muscle plasma membranes (MacKinnon & Miller, 1988) have suggested that CTX physically plugs the channels' externally facing pore. There are three mechanisms by which maxi-K^+ channel activity can be modulated: pH_i, Ca^{2+} (Christensen & Zenthen, 1987) and protein phosphorylation (Reinhart et al., 1991). Patch-clamp studies have shown that acidification at a fixed Ca^{2+} concentration decreases maxi-K^+ channel activity in Necturus choroid plexus (Christensen & Zenthen, 1987), pancreatic β cells (Cook et al., 1984) and cultured renal medullary thick limb cells (Cornejo et al., 1989). Alkalinization in a physiological range will increase maxi-K^+ channel activity (Copello et al., 1991). Our ^{86}Rb efflux data and cell volume experiments suggest that the methyl-isobutyl-amiloride sensitive alkalinization precedes the activation of the CTX-sensitive K^+ channel after 5-7% swelling in enterocytes. The increased rates of ^{86}Rb efflux in addition to RVD were inhibited by either the amiloride derivative or by Na^+-free medium, both conditions which blocked the alkalinization of pH_i. When sufficient NH_4Cl was added to alkalinize the cells in spite of this inhibition, RVD proceeded. When CTX was added to the amiloride treated cells which had also received NH_4Cl to alkalinize them, this RVD was prevented. If the amiloride-sensitive alkalinization did not precede the activation of the CTX-sensitive K^+ channel, neither Na^+ free medium or the amiloride derivative would have influenced the increased rate of ^{86}Rb efflux stimulated by 0.93 x isotonic dilution. Therefore, we conclude that the amiloride-sensitive alkalinization which occurs with the modest 5 to 7% volume increase of cells is required for the activation of CTX-sensitive K^+ channels utilized for RVD in jejunal villus epithelium.

In the current experiments, volume increases of ≥ 12% after substantial hypotonic dilution were insensitive to CTX, yet, directly stimulating an alkalinization of pH_i in these cells resulted in volume changes that were CTX-sensitive. However, in the absence of extracellular Ca^{2+}, increasing pH_i of cells which had a volume increase of 15% did not result in CTX-sensitivity. We assume that maxi-K^+ channels require an increase in $[Ca^{2+}]_i$ for activation after cell swelling (Latorre et al., 1989), and reports of other epithelial cell types have suggested that maxi-K^+ channels are responsible for K^+ efflux during RVD (Christensen, 1987; McCarty and O'Neil, 1992; Okada et al., 1990; Suzuki et al., 1990; Foskett, 1994; Park et al., 1994). While we have not quantitated $[Ca^{2+}]_i$ in the current experiments, we have shown RVD after modest 5-7% and after larger 15 to 20% volume increases requires mobilization of extracellular Ca^{2+} and is prevented by loading cells with a calcium buffer, suggesting that $[Ca^{2+}]_i$ increases were occurring with both amounts of swelling (MacLeod et al., 1992). Because depletion of extracellular Ca^{2+} prevented CTX-sensitive volume changes in spite of a substantial alkalinization of pH_i, our results suggest that in villus cells only if there is both an increase in $[Ca^{2+}]_i$ and an alkalinization of pH_i will a CTX-sensitive K^+ channel be activated. Thus, with large volume increases, $[Ca^{2+}]_i$ increases but the pH_i acidifies (MacLeod et al., 1994A) and the subsequent RVD is CTX-insensitive. A relationship emerges between the size a villus cell swells after suspension in hypotonic media, the sensitivity of subsequent RVD to amiloride derivatives (Fig. 8A) and the sensitivity of RVD to CTX (Fig. 8B). The sensitivity of volume regulation to amiloride

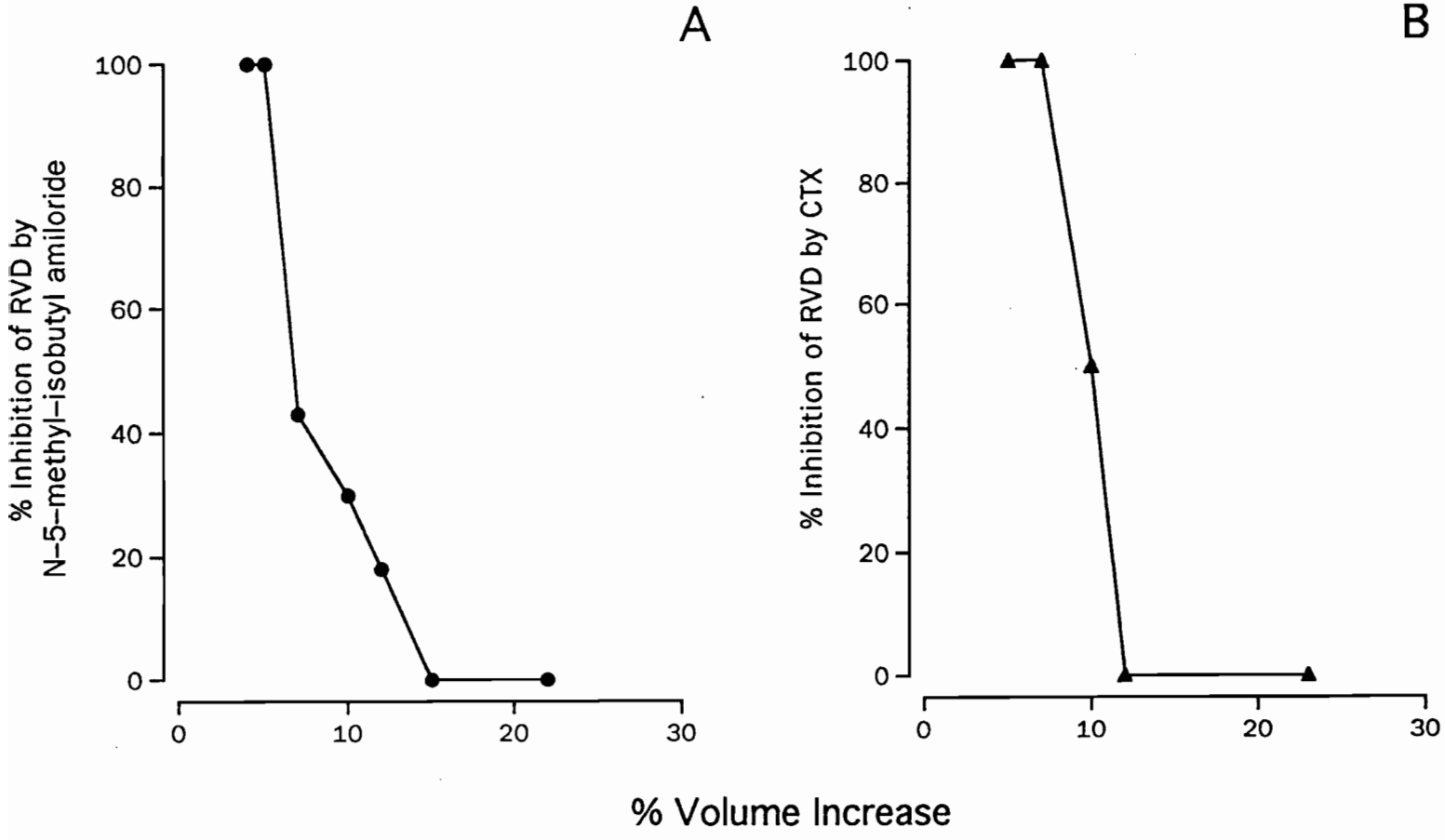
derivatives is equivalent to amiloride-sensitive alkalinization of pH_i observed when the villus cell swells. Clearly, the two sensitivity curves are superimposable.

The cell permeant inhibitor of cAMP-dependent protein kinase A, Rp-cAMPS (Rothermel et al., 1988) prevented cAMP induced volume changes (MacLeod et al., 1994B) but had no effect on RVD after 0.93 x isotonic dilution. This finding suggests that the CTX-sensitive K^+ channel required for `physiological' RVD is not activated by protein kinase A. Previously we have reported that different inhibitors of protein kinase C have no effect on RVD after a 0.95 x isotonic dilution (MacLeod et al., 1992B). Together these results suggest neither PKA nor PKC activate the CTX-sensitive K^+ channel responsible for `physiological' RVD in the villus cells.

Our results indicate a direct relationship between the extent of villus cell swelling and the class of K^+ channel activated for RVD. The linkage between the two is the change in pH_i which occurs during the volume increase. 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 and 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 et al., 1988A,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 et al., 1995). Our current findings contrast with experiments using proximal

convoluted kidney tubules, where a HCO_3 dependent SITS sensitive alkalinization of pH_i was observed after a standard, 0.7 x isotonic challenge. In these studies, it was concluded that alkalinization of pH_i was not the sole factor responsible for increasing the K^+ conductance required for RVD (Beck et al., 1992). Yet with jejunal villus cells, a sharp distinction exists with the extent of cell swelling and the class of K^+ channel activated. Larger volume increases observed after `standard' 0.8 x isotonic challenge are followed by RVD insensitive to CTX, while modest 5-7% volume increases, which result in amiloride-sensitive alkalinizations of pH_i are followed by RVD which is blocked by CTX.

Figure 8: Differential sensitivity of RVD to extracellular Na+ (A) or CTX (B) as a function of how large a villus cell swells in response to hypotonic dilution. Sensitivity to N-5-methyl-isobutyl amiloride (1 μM) superimposable on curve A. Mean of 5 experiments performed in duplicate are illustrated.


A
B
% Inhibition of RVD by N-5-methyl-isobutyl amiloride
% Inhibition of RVD by CTX
100
80
60
40
20
0
0
10
20
30
% Volume Increase

Our current results suggest a causal relationship between the alkalinization of pH_i and the activation of CTX-sensitive K^+ channels required for volume regulation in jejunal villus cells exposed to 0.93 x isotonic medium. Because the resulting modest volume increase duplicates the size these cells swell during Na^+-solute absorption, and because the consequent RVD is CTX-sensitive, we suggest there is a physiologically relevant size increase which is distinctive to Na^+-absorbing villus epithelial cells. Thus, the size these epithelial cells swell is an important determinant of the signal transduction reponsible for activating the K^+ channels required for subsequent volume regulation.

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A modest (5 to 7%) volume increase of a villus cell causes a G-protein to activate Na^+/H^+ exchange (NHE) which results in a transient alkalinization of pH_i. A charybdotoxin-sensitive, Ca^{2+}-activated K^+ channel absolutely requires this alkalinization of pH_i for activation to allow volume regulation to proceed. With greater volume increases the pH_i acidifies, partially due to NHE activity, and the K^+ channel activated for this volume regulation is charybdotoxin-insensitive. To understand more about the signalling characteristics of the charybdotoxin-insensitive K^+ channel activated because of greater volume increases, experiments were designed to answer the following questions: Do tyrosine kinase or Ca^{2+}/calmodulin II inhibitors influence RVD after modest or large volume increases? Do inhibitors of phosphoprotein phosphatases influence the rate or direction of RVD? Will phosphatase inhibitors by-pass inhibition of RVD caused by alterations in the K^+ chemical gradient? To answer these questions we made volume determinations of cells after modest or large volume increases in the presence of different kinase or phosphatase inhibitors.

Chapter Five

Signal transduction of K^+ channel activation for Regulatory Volume Decrease depends on extent of Epithelial Cell Swelling

Summary

To test the hypothesis that the extent of epithelial cell swelling is a determinant of the signal transduction responsible for activating the K^+ channels for Regulatory Volume Decrease (RVD), we determined the effect of 1-N-0-bis (5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl-4-phenyl-piperazine (KN-62) an inhibitor of Ca^{2+}/calmodulin kinase II, on RVD of guinea pig villus enterocytes. Cell volume was assessed by electronic cell sizing. Following a 0.70 x isotonic dilution, RVD was blocked by KN-62 (50 μM). Gramicidin (0.5 μM) bypassed inhibition suggesting the K^+ but not the Cl^- channel was affected by KN-62. Modest volume increases of 7%, which duplicated the extent of volume increase these cells undergo with Na^+- solute absorption, were followed by RVD not influenced by KN-62. Both okadaic acid and calyculin A, inhibitors of phosphoprotein phosphatases 1 and 2A, accelerated RVD after 20% volume increases, and in K^+ rich medium, okadaic acid (1 μM) caused sustained secondary cell swelling. When these volume changes were rate-limited by the volume-sensitive Cl^- channel, okadaic acid had no effect. The inhibition of RVD generated by 10 or 20 mM extracellular K^+ was bypassed by addition of okadaic acid. An inhibitor of tyrosine kinase, genistein (100μM) had no effect on RVD after 0.7 x isotonic dilution. We conclude the charybdotoxin-insensitive K^+ channel utilized for RVD after large `non-physiological' volume increases is activated by phosphorylation mediated by Ca^{2+}/calmodulin kinase II.

Our results suggest that different signal transduction pathways exist for activating K^+ channels required for RVD and that these pathways are determined by the extent of cell swelling.

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, 1991A,B): the signal transduction responsible for activating the K^+ channel is absolutely dependent on the extent of the volume increase that occurs (MacLeod et al., 1994A). Following `standard' hypotonic dilution (0.70 x isotonic) commonly used in volume regulation protocols (reviewed in Hoffmann & Dunham, 1995; Strange, 1994), villus cells swell ≈ 15% and calmodulin antagonists prevent activation of the K^+ channel. This response contrasts with the response to volume increases of 5 to 7% occurring because of Na^+-solute absorption, where the K^+ channel activated is charybdotoxin (CTX)-sensitive and not influenced by the same calmodulin antagonists (MacLeod et al., 1992).

As phosphorylation and dephosphorylation are known to control the activity of certain volume-sensitive cotransporters (O'Donnell et al, 1995) and since high conductance, Ca^{2+}-activated (maxi-K^+) channels may be modulated by phosphorylation (Levitan, 1994), we assessed villus cell volume changes in response to different kinase and phosphatase inhibitors. We found that these inhibitors had a distinct effect on volume regulation which was contingent upon the size the villus cells swelled after hypotonic dilution. Our results support the concept that while the same osmolyte (K^+) is lost for volume regulation after modest or large volume increases in these cells, different signal transduction pathways resulting in K^+ channel activation are operative conditional on the extent of cell swelling.

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 as previously described (MacLeod & Hamilton, 1990). Isolated cells were resuspended at 0.8 to 1.5 x 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%, 3 hr after suspension in medium. Cell volume was measured using a Coulter Counter (Zm) with an attached Channelyzer (C-256) as previously described (MacLeod & Hamilton, 1991A,B). Villus cell volume measured electronically over a range of tonicities correlated positively (r=0.976) with direct measurements of cell water (MacLeod & Hamilton, 1991B). Relative cell volume was determined as the ratio of cell volume under experimental conditions to volume in isotonic medium immediately before challenge.

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). K^+ rich medium and medium containing 10 and 20 mM K had NaCl isosmotically replaced by KCl. Na^+-free medium was prepared by isosmotically 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) calyculin A from Boehringer Mannheim (Montreal, P.Q.) and $NMDG^+$ from Aldrich (Milwaukee, WI). Gramicidin and genistein were from Sigma Chemical (St. Louis, MO). RPMI-1640 (10x) medium was from GIBCO/BRL (Burlington, Ont.).

Statistics

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

Results

Effect of KN-62, 1-[N-0-bis(5-isoquinoline-sulfonyl)-N-methyl-L-tyrosyl-4-phenyl-piperazine on RVD after marked or modest hypotonic dilution

We determined the effect of the inhibitor of Ca^{2+}-calmodulin kinase II, KN-62 (50μM), on RVD by hypotonically (0.7 x isotonic) diluting suspended villus enterocytes and measuring the subsequent volume response (Fig. 1). In the presence of KN-62 and this `standard' hypotonic medium the villus cells swelled, but the subsequent RVD was prevented (Fig. 1A). Gramicidin (0.5μM) allowed a complete bypass of this inhibition of RVD by KN-62 (final rel. vol.: 0.95 ± .02 vs 1.14 ± .01, $p<0.001$) suggesting that it is the volume-sensitive K^+ channel which is the target of Ca^{2+}-calmodulin kinase II. When we duplicated the amount of cell swelling that occurs when these cells absorb D-glucose or L-alanine, by a 0.93 x isotonic dilution (Fig. 1B), KN-62 did not inhibit the subsequent RVD.

Effect of okadaic acid or calyculin A on RVD after marked (0.7 x isotonic) dilution

If the volume-sensitive K^+ channel is activated by phosphorylation, presumably 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 x 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 ± .01, $p<0.05$, n=6). Calyculin A, another inhibitor of phosphoprotein phosphatases I and 2A (Ishihara, et al., 1989) accelerated RVD of the cells in a dose-responsive manner. We compared the $t_{0.5}$ of RVD of cells treated with calyculin A or okadaic acid. Calyculin A was more potent than okadaic acid in accelerating volume reduction (EC_{50} 100 nM vs ~ 750 nM).

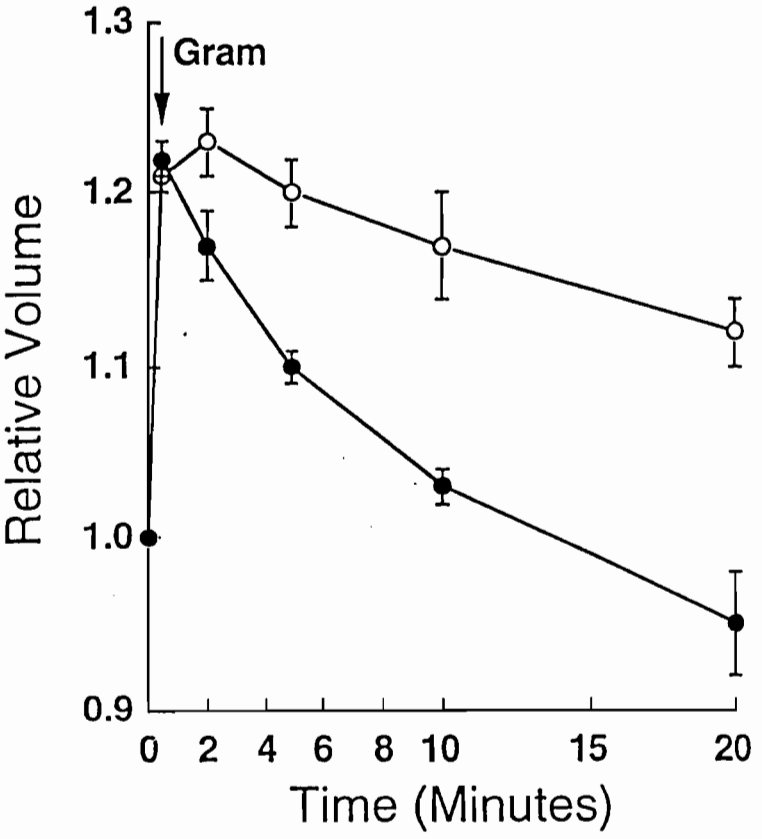
Effect of okadaic acid on volume changes directed by extracellular K^+

Because the RVD response to extensive cell swelling is determined by the activation of both volume-sensitive K^+ and Cl^- conductances (MacLeod & Hamilton, 1991A) we sought additional evidence that it was the volume-sensitive K^+ conductance which was influenced by the phosphatase inhibitor by suspending cells in K^+ rich medium (Fig. 3). The okadaic acid studies described above were repeated using medium containing 140 mM K^+ rather than 3 mM to negate the K^+ gradient (MacLeod & Hamilton, 1991A). As illustrated in Figure 3A, not only was RVD inhibited under these conditions, but K^+ influx ensued causing secondary cell swelling. The final relative volume of the cells exposed to okadaic acid was larger than untreated controls (1.27 ± .01 vs 1.22 ± .02, $p<0.05$, n=5).

Figure One:

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

A


1.3
1.2
1.1
1.0
0.9
Gram
Relative Volume
0
2
4
6
8
10
15
20
Time (Minutes)

B

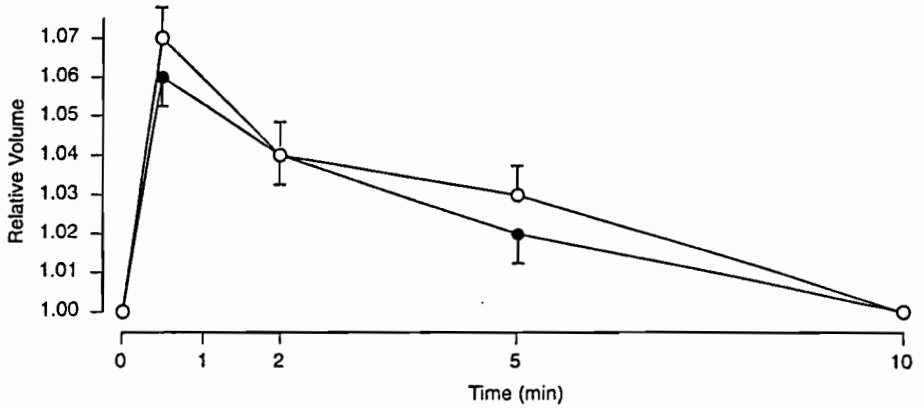
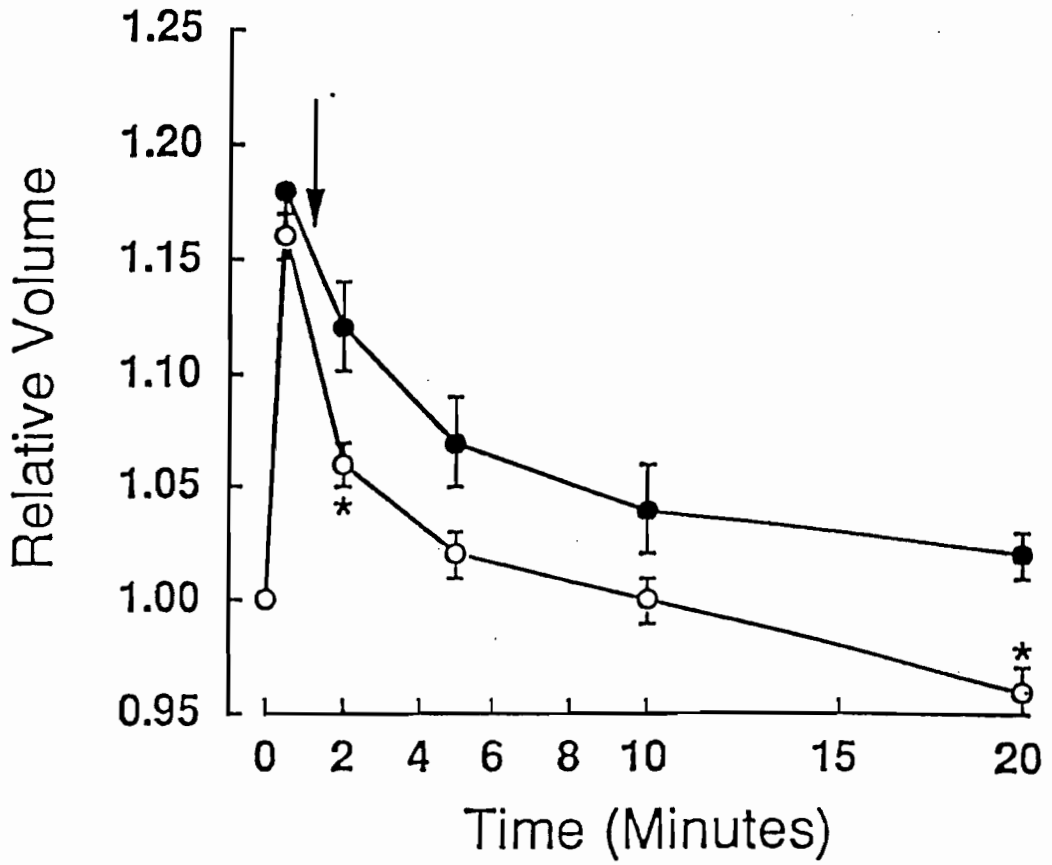

Relative Volume
1.07
1.06
1.05
1.04
1.03
1.02
1.01
1.00
0
1
2
5
10
Time (min)

Figure Two:

Effect of okadaic acid A on RVD. O, okadaic acid (1μM), 0.7 x isotonic; ●, 0.7 x isotonic. Experiment performed in Na^+-free medium. *$p<0.05$. n=5. Volume measured electronically, expressed relative to isotonic control.


1.25
1.20
1.15
1.10
1.05
1.00
0.95
Relative Volume
*
*
0
2
4
6
8
10
15
20
Time (Minutes)

To assess whether the volume-activated Cl^- channel was influenced by okadaic acid, we added gramicidin (0.5) to cells after 0.7 x isotonic dilution in the same K^+ rich medium (Fig. 3B). In the presence of gramicidin, the volume-sensitive Cl^- channel is rate-limiting for any volume change (MacLeod & Hamilton, 1991A). Under these conditions, okadaic acid had no effect on cell volume. We then varied the extracellular K^+ concentration and assessed whether okadaic acid influenced the inhibition of RVD (Fig. 4). When $[K^+]_o$ was 20 mM, RVD was incomplete. The final relative volume of cells was greater compared with controls suspended in $[K^+]$ of 3 mM (1.15 ± 0.02, $p<0.05$, Fig. 4A). Okadaic acid (1μM) added after 0.5 x isotonic dilution of cells 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 final relative volume in comparison with controls (final rel. vol.: 1.04 ± .02 vs 1.15 ± 0.02, $p<0.05$). When the extracellular $[K^+]$ was 10 mM, okadaic acid had no effect on the initial rate of cell shrinkage, but caused the cells to reach a smaller final relative volume (Fig. 4B). The final relative volume of these cells was smaller than untreated controls (1.00 ± .02 vs 1.07 ± .02, $p<0.05$, n=5). Together, these data suggest that the activity of the volume-sensitive K^+ channel in villus cells may be modulated by an inhibitor of phosphoprotein phosphatases.

Figure Three:

Effect of okadaic acid on secondary swelling in K^+ rich medium. (A) ●, 0.5 x isotonic; O, okadaic acid (1μM), 0.5 x isotonic. (B) O, okadaic acid, 0.5 x isotonic, gramicidin (0.5μM); ●, 0.5 x isotonic, gramicidin (0.5 μM). n=6. Volume measured electronically, expressed relative to isotonic control.

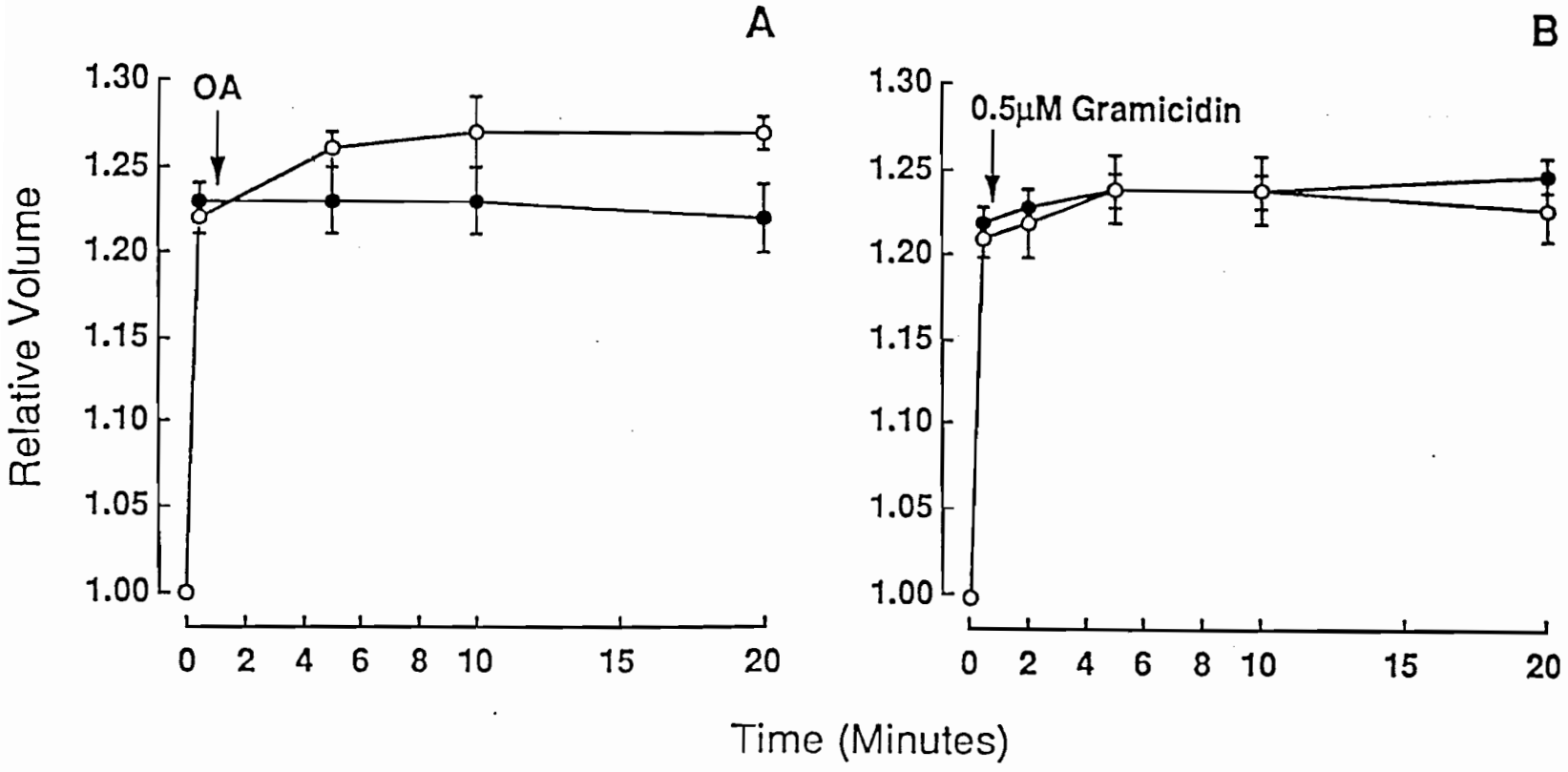
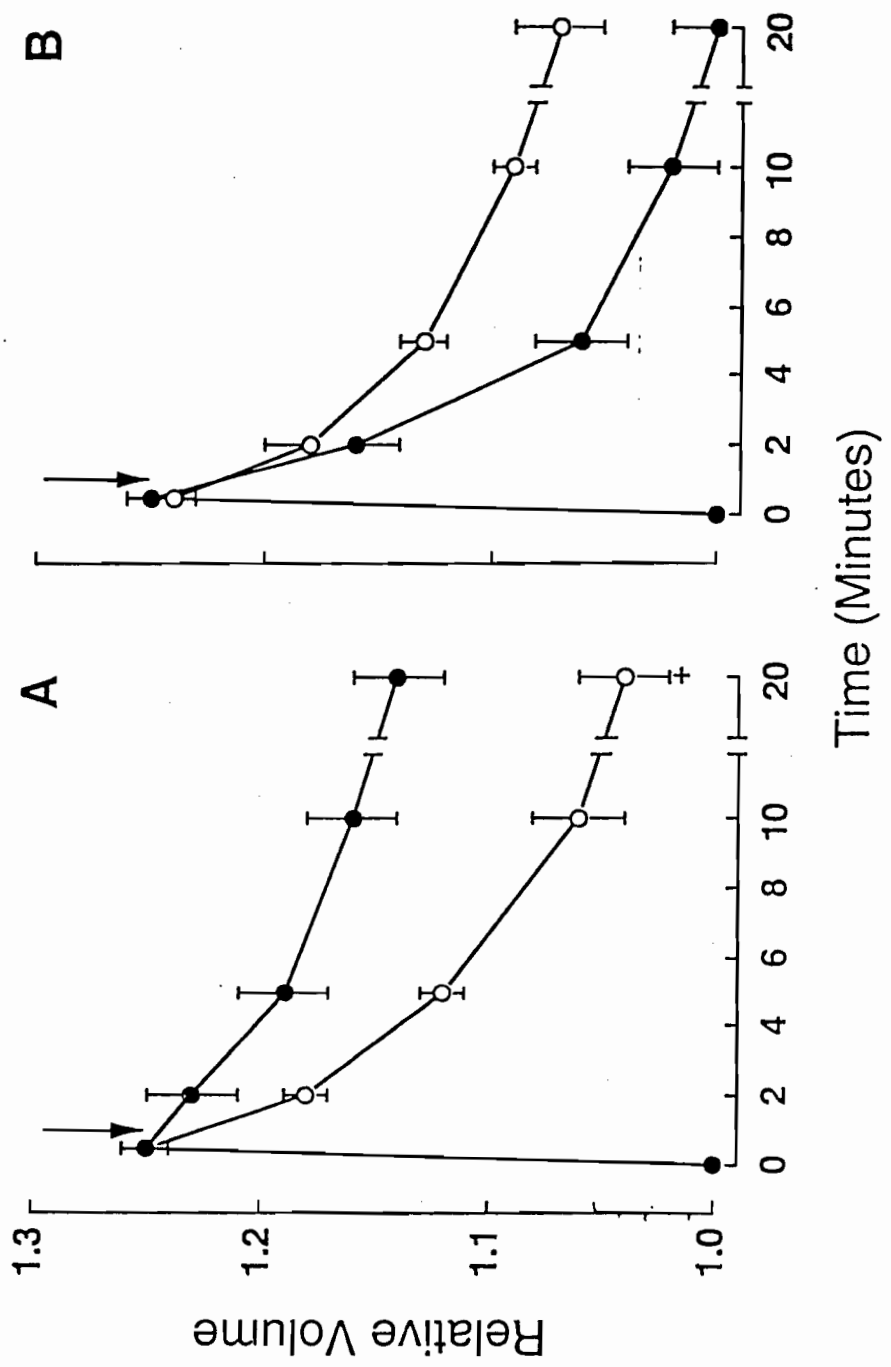

A
B
OA
0.5μM Gramicidin
Relative Volume
1.30
1.25
1.20
1.15
1.10
1.05
1.00
0
2
4
6
8
10
15
20
Time (Minutes)

Figure Four:

Okadaic acid bypasses inhibition of RVD by extracellular K^+. (A) ●, 20 mM K^+, 0.5 x isotonic; O, okadaic acid (1 μM) + 20 mM K^+, 0.5 x isotonic. (B) O, 10 mM K^+, 0.5 x isotonic, ●, okadaic acid + 10 mM K^+ + 0.5 x isotonic. n=5. Volume measured electronically, expressed relative to isotonic control.

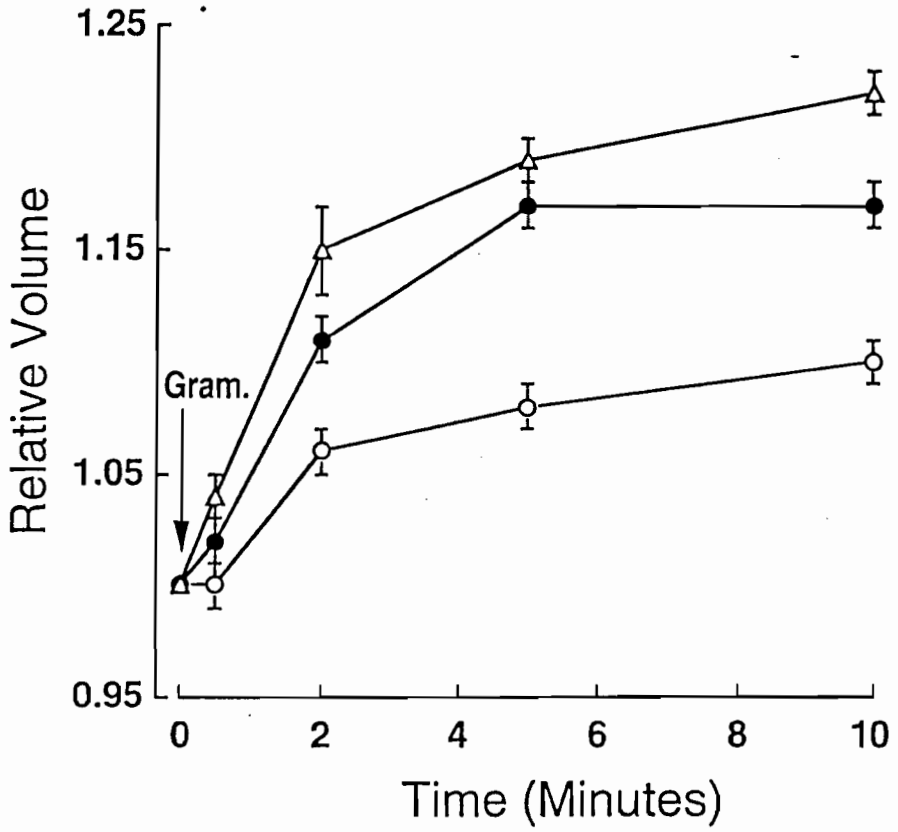

A
B
Relative Volume
1.3
1.2
1.1
1.0
0
2
4
6
8
10
20
Time (Minutes)

Effect of okadaic acid on isotonic volume changes induced by 12-0-tetradecanoyl-phorbol 13-acetate (TPA)

We measured the effect of okadaic acid (1μM) on villus cell volume changes when anion permeability was rate-limiting for these volume responses (Fig. 5). The addition of the cation ionophore gramicidin (0.5 μM) to villus cells in isotonic Cl^- containing, but not Cl^- free medium, resulted in cell swelling which was accelerated by phorbol ester or synthetic diacylglycerol (MacLeod, Lembessis & Hamilton, 1992A). These results suggest that villus cells possess a Cl^- channel which can be activated by protein kinase C. Accordingly we compared the effect of okadaic acid on phorbol ester (TPA) stimulated volume changes in cells permeabilized by gramicidin. TPA (10^{-8}M) addition caused an increase in the initial rate of cell swelling (% vol increase • $cell^{-1}$ • min^{-1}: 3.5 ± 0.2 vs 2.5 ± 0.2, $p<0.05$) and an increase in the final relative volume of cells compared with cells treated with gramicidin alone (final rel. vol.: 1.16 ± 0.01 vs 1.08 ± 0.01, $p<0.05$). Okadaic acid and TPA caused a faster initial rate of cell swelling (% vol. increase • $cell^{-1}$ • min^{-1}: 5.5 ± 0.5 vs 3.5 ± 0.2, $p<0.05$) than TPA alone. Cells treated with okadaic acid and TPA swelled to a larger volume compared with cells treated with TPA alone (final rel. vol. 1.2 ± 0.01 vs 1.16 ± 0.01, $p<0.05$). When phosphorylation mediates the activation of anion permeability in these cells, the inhibitor of phosphoprotein phosphatases, okadaic acid, accentuates this effect.

Figure Five:

Okadaic acid stimulates phorbol ester stimulated cell swelling. O, gramicidin (0.5 μM); ●, TPA (10^{-8}M) + gramicidin; Δ, okadaic acid (1μM) + TPA + gramicidin. Experiment performed in isotonic NaCl medium. n=6. Volume measured electronically, expressed relative to isotonic untreated cells.

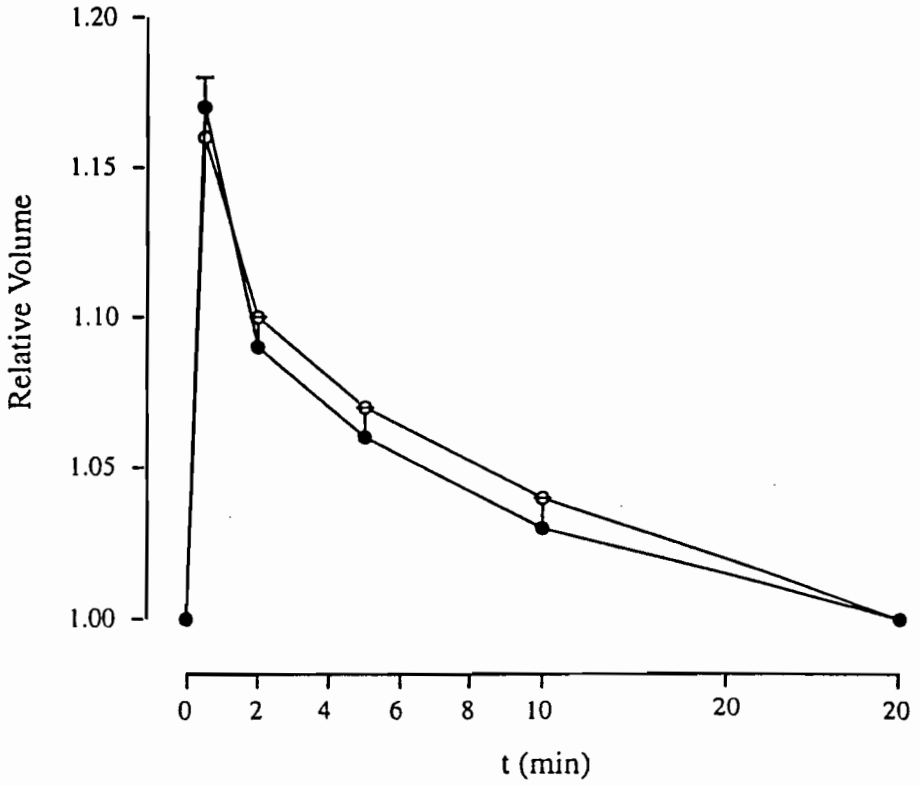

1.25
1.15
1.05
0.95
Relative Volume
Gram.
0
2
4
6
8
10
Time (Minutes)

Effect of tyrosine kinase inhibitors on RVD

The tyrosine kinase inhibitor, genistein (100 μM) (Fig. 6) had no effect on RVD after suspension of the cells in 0.70 x 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). Comparable results were observed at 200 μM genistein.

Figure Six:

Effect of genistein on RVD. ●, genistein (100μM), 0.7 x isotonic; 0, 0.7 x isotonic. Genistein at 200 μM had no effect. n=6. Volume measured electronically, expressed relative to isotonic control.


1.20
1.15
1.10
1.05
1.00
Relative Volume
0
2
4
6
8
10
20
20
t (min)

Discussion

Previously we have shown that calmodulin antagonists W-7 and W-13 prevent RVD after large volume increases but they have no effect on RVD after modest `physiological' volume increases caused by L-alanine absorption (MacLeod et al., 1992B). Because the inhibition of RVD by calmodulin antagonists was bypassed with the cation ionophore gramicidin, we suggest 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. Because the inhibitory effects of KN-62 could also be bypassed by providing a surrogate K^+ channel, our results suggest that it is the K^+ and not the 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 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.

After extensive volume increases, RVD has been shown to be prevented by Ba^{2+}, quinine and apamin, indicating that a high conductance Ca^{2+}-activated K^+ (maxi K^+) channel is activated for these volume changes (MacLeod & Hamilton, 1991A). In recent experiments we have shown that RVD after 12-22% swelling was not affected by charybdotoxin (CTX), a potent inhibitor of some maxi-K^+ channels,

while the RVD after modest 'physiological' cell swelling (5 to 7%) was CTX-sensitive (MacLeod et al., 1992B, 1994B). Thus, while large and modest volume increases rely on maxi-K^+ channels for RVD, these channels are distinguished by their sensitivity to CTX and an inhibitor of CaMKII. In keeping with these results, others have reported that two maxi-K^+ channels which have the same Ca^{2+} and voltage sensitivities, are distinguished by sensitivity and insensitivity to CTX (Reinhart et al., 1989). A large conductance (~130 pS) K^+ channel was identified in isolated guinea pig villus enterocyte membrane patches which was outwardly-rectifying and insensitive to both Ba^{2+} and changes in Ca^{2+} concentration (Mintenig et al., 1992). The CTX or calmodulin sensitivity of this channel were not assessed, presumably because of its insensitivity to calcium. In contrast, we have shown that RVD after both large and modest (5-7%) cell swelling is exquisitely sensitive to extracellular Ca^{2+}, and that irrespective of the extent of the volume increase, buffering increases in $[Ca^{2+}]_i$ prevented the subsequent volume regulation (MacLeod et al., 1992). These results lead us to propose that with large volume increases, an increase in $[Ca^{2+}]_i$ occurs. This putative $[Ca^{2+}]_i$ increase activates calmodulin, and the Ca^{2+}-calmodulin complex activates CaMKII. The current experiments suggest the CTX-insensitive K^+ channel which is required for RVD is activated by this kinase.

We found that Okadaic acid (OA) a membrane permeant inhibitor of protein phosphatases 1 and 2A accelerated the rate of RVD after large volume increases and caused secondary volume increases of the villus cells in K^+ rich medium. Calyculin A (Ishihara et al., 1989) which inhibits phosphatases 2A with a similar

potency to okadaic acid but is a more potent inhibitor of phosphatase 1, was about seven times more potent in accelerating RVD than OA. It has been established that OA does not affect protein kinase C, cAMP-dependent protein kinase or CaM kinases at concentrations up to 5 μM (Bialojau and Takai, 1988; Cohen, 1989; Hardie et al., 1991). OA added to resting rat thymic lymphocytes in isotonic medium stimulated the NHE-1 isoform of Na^+/H^+ antiport with increased phosphorylation of the antiport, indicating that the level of constitutive phosphorylation of the antiport is related to its activity (Bianchini et al, 1991). By using Na^+-free medium in our experiments we circumvented activation NHE-1 by OA in the villus cells. If OA was activating NHE-1, the CTX-sensitive K^+ channel is activated because of the alkalinization of pH_i, and RVD after a large volume increase becomes sensitive to CTX (MacLeod et al., 1994B). The activity of $NaKCl_2$ cotransport in duck erythrocytes is stimulated by OA, again, consistent with the view that the activity of this system requires constitutive phosphorylation (Pewitt et al., 1990; Palfrey, 1994). Studies of shark rectal gland have suggested that hyperosmotic activation of $NaKCl_2$ cotransport also occurs via a phosphorylation mechanism (Lytle and Forbush, 1992). OA and other phosphoprotein phosphatase inhibitors such as fluoride, vanadate and calyculin A have been used to infer that hypotonically activated KCl cotransport or a putative cofactor is regulated by phosphorylation. In studies of human (Kaji and Tsuketani, 1991) or rabbit (Jennings and Al-Rohil, 1990, Jennings and Schulz, 1991) red cells, OA increases the lag between initial cell swelling and the activation of KCl cotransport, whereas the rate of deactivation of KCl cotransport observed in shrinking the cells back to

their normal volume is not influenced by these inhibitors. These results suggest that activation of KCl cotransport by red cell swelling involves a net dephosphorylation. In the current experiments when volume changes were rate-limited by the K^+ gradient, OA either bypassed inhibition of RVD or caused secondary swelling. When volume changes were rate-limited by the volume-sensitive Cl^- channel, OA had no effect. Under conditions where volume changes were rate-limited by a protein kinase C activated Cl^- channel (MacLeod et al., 1992A), OA accelerated and potentiated these volume changes. Therefore, because of these distinct effects of OA and calyculin A on volume regulation after large volume increases, we conclude that the CTX-insensitive K^+ channel required for RVD is activated by phosphorylation.

Since genistein, a tyrosine kinase inhibitor (Akiyama and Ogawara, 1991) had no effect on RVD after large cell swellings, we suggest that tyrosine kinase is not responsible for activating the KN-62 sensitive K^+ channel required for RVD. Our current data from villus enterocytes freshly isolated from mature guinea pig differ from the findings of others using the fetal derived intestine 407 cell line (Tilly et al., 1993). Herbimycin A and genistein were reported to inhibit both hypotonically activated ^{125}I and ^{86}Rb efflux suggesting that cell swelling triggered tyrosine kinase activity. In fact, our volume data using the CaMKII inhibitor and OA are consistent with the proposition that substantial villus cell swelling either stimulates kinase activity or inhibits phosphatase activity. While we have not measured enzyme activities in this study, the differential sensitivity of RVD after modest swelling compared with larger cell swellings to KN-62 implicates activation of CaMKII in

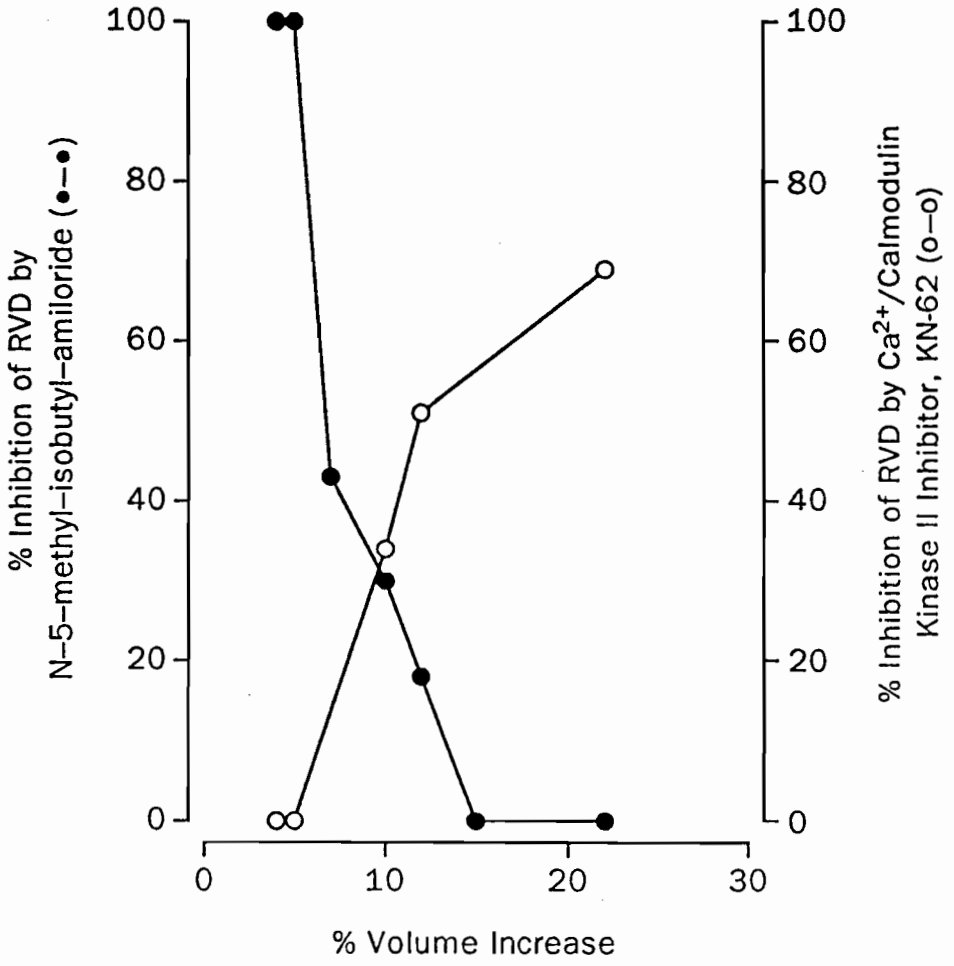
extensive villus cell swellings. Molecular characterization of high conductance Ca^{2+}-activated (maxi-K^+) K^+ channels has revealed that they and their regulation are far more diverse than previously suspected (Latorre et al., 1989; Atkinson et al., 1991; Butler et al., 1993). For example, Drosophila Slo maxi-K^+ channels expressed in Xenopus oocytes were shown to be activated by an endogenous protein kinase A-like kinase which remained functionally associated with the channels (Esguerra et al., 1994). Accordingly, we speculate that in villus enterocytes, the CTX-insensitive, KN-62 sensitive K^+ channel which is utilized for RVD after large volume increases may be functionally associated with CaMKII.

Modest `physiological' cell swelling, which duplicates the size these cells swell when they absorb L-alanine or D-glucose, results in an increase of $pH_i$ which is blocked by an N-5-alkyl amiloride derivative. The RVD after this `physiological' cell swelling was prevented by the amiloride derivative (MacLeod et al, 1994B). This intracellular alkalinization of pH_i is a determinant of the activation of a CTX-sensitive K^+ channel required for RVD (MacLeod et al., 1994A). In contrast, larger cell swellings cause the pH_i to acidify, and the subsequent RVD is not affected by the N-5-alkyl amiloride derivative. In the present studies (Fig. 7), the sensitivity of RVD to the CaMKII inhibitor is a mirror image of the sensitivity of RVD to N-5-methyl isobutyl amiloride. The sensitivity of RVD to CTX as a function of how large the villus cell swells is superimposable on the sensitivity of RVD to the amiloride derivative. A presumption of many volume regulatory studies is that the same signal transduction pathways are responsible for activating osmolyte loss, irrespective of the size a cell initially swells (Hoffmann and Dunham, 1995; Strange,

1994). Some reports have suggested differences in volume regulatory behaviour based on the amount of swelling after hypotonic dilution; 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 and Cahalan, 1995; Sarkadi et al., 1984; Hallows and Knauf, 1994). Our current results clearly suggest that activation of the K^+ channel responsible for RVD requires a calmodulin kinase when villus cells swell to amounts greater than that observed during Na^+-solute absorption. We conclude the size an epithelial cell swells is a determinant of the signal transduction responsible for activating the K^+ channel required for volume regulation.

Figure Seven:

Differential sensitivity of RVD to KN-62 or N-5-methyl-N-isobutyl(-amiloride (MIA) as a function of how large a villus cell swells in response to hypotonic dilution. Sensitivity of RVD to Na^+ free medium or charybdotoxin superimposable on MIA curve. Mean of five experiments performed in duplicate are illustrated.


100
80
60
40
20
0
0
10
20
30
% Volume Increase
% Inhibition of RVD by
N–5–methyl–isobutyl–amiloride (•–•)
% Inhibition of RVD by Ca2+/Calmodulin
Kinase II Inhibitor, KN-62 (o–o)

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Chapter Six

CONCLUSIONS

From the evidence presented, I conclude:

1. Modest volume increases of villus cells, which duplicate the extent of the volume increase experienced by these cells during Na^+-solute absorption, result in an alkalinization of pH_i.

2. The source of this alkalinization is Na^+/H^+ exchange (NHE).

3. NHE-1 is the isoform of NHE which is activated by the modest swelling.

4. NHE-1 activation is not due to cellular acidification.

5. A pertussis-toxin-insensitive heterotrimeric GTP-binding protein (G-protein) mediates the activation of NHE-1 during modest volume increases.

6. Intact microfilaments are required for the G-protein to activate NHE-1.

7. It is the alkalinization of pH_i due to the activated NHE-1, not the increased Na^+ influx, which is the requirement for Regulatory Volume Decrease (RVD) after the modest volume increase.

8. A charybdotoxin (CTX)-sensitive, Ca^{2+}-activated K^+ channel which requires intracellular alkalinization for activation, is the source of K^+ loss for RVD after the modest volume increase.

9. Larger volume increases after substantial hypotonic dilution cause the pH_i to acidify.

10. NHE is inhibited with greater volume increases, which contributes to the net acidification of pH_i.

11. The K^+ channel activated for RVD after greater volume increases is CTX-insensitive, and requires Ca^{2+}-calmodulin kinase II for activation.

12. The size these epithelial cells swell determines whether NHE-1 is activated or inhibited. The change in pH_i is a determinant of the class of K^+ channel activated for the subsequent volume regulation.

In the remainder of this chapter, I shall briefly review the evidence presented in Chapters Two to Five which support the conclusions listed above. The diagram illustrated in Fig. 1 models the findings described in Chapters Two, Three and Four, while the diagram illustrated in Fig. 2 combines findings from Chapter Two with Chapter Five. After reiterating my conclusions, I shall briefly describe two directions for further research which use villus epithelial cells to determine the nature of the "volume sensor".

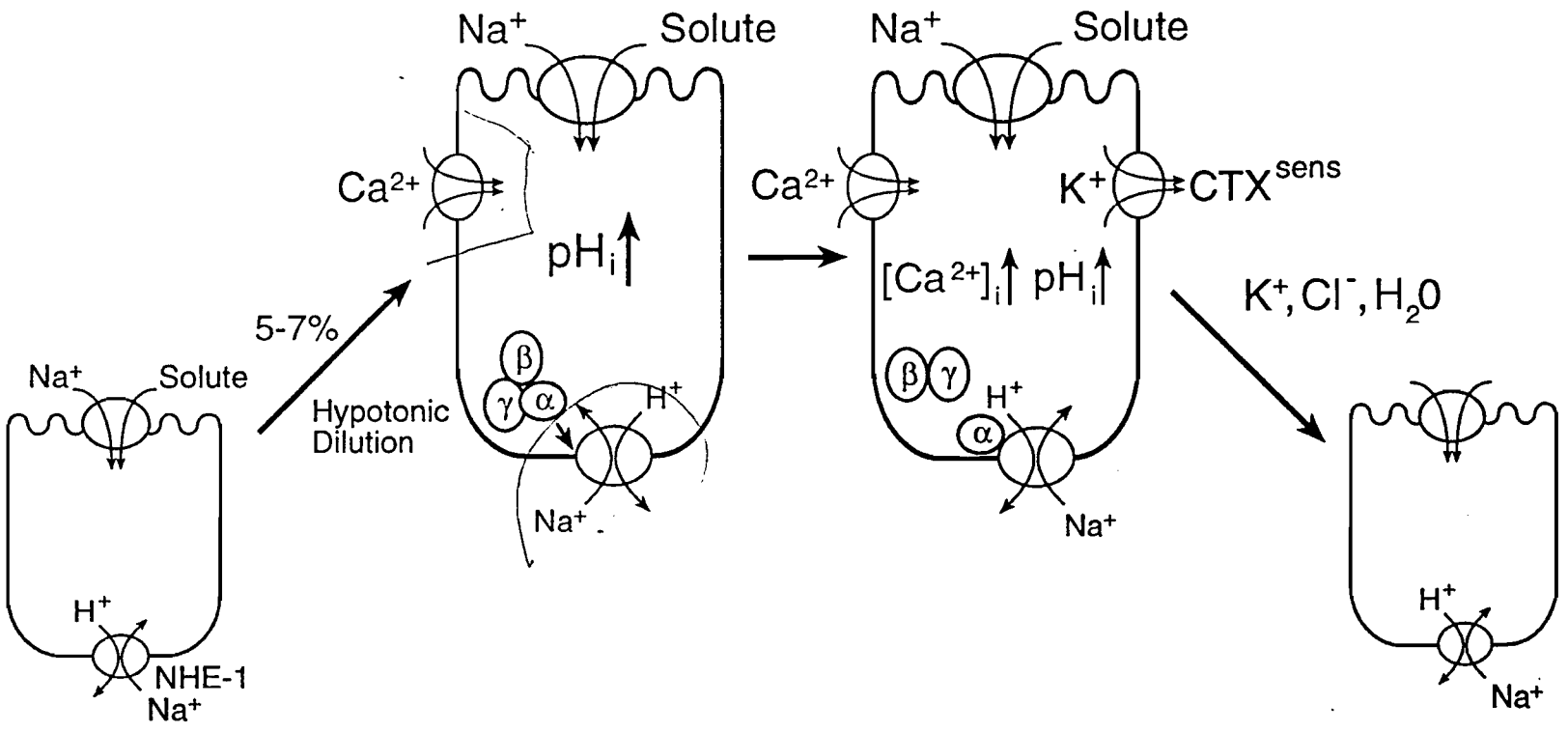
1. Signal transduction of volume regulation after modest volume increases

Modest, swelling of the villus cells to the same size they reach during Na^+-solute absorption causes the pH_i to alkalinize (Fig.1). This change in pH_i was blocked by low concentrations of a N-5-alkyl-amiloride derivative, consistent with increases of Na^+/H^+ activity. The subsequent volume regulation, RVD, required extracellular Na^+ and was also prevented by the N-5-alkyl-amiloride derivative (MIA). Using non-amiloride derivatives and alkyl-amiloride derivatives the order of potency of inhibiting RVD was: MIA > DMA > cimetidine > clonidine. Because only NHE-1 is more sensitive to cimetidine than clonidine, I conclude that NHE-1 was the isoform of the exchanger responsible for the pH_i change accompanying `modest' cell swelling. The rate of MIA sensitive pH_i recovery from an acid load was measured in media of different tonicities and determined to have the following hierarchy:

5% Hypotonic > Isotonic > 30% Hypotonic. eq. 1

Thus, the intracellular acidification which occurs with any volume increase was not the mechanism of activation of NHE-1 for RVD.

Figure One: The findings of the experiments described in Chapters Two, Three, and Four are illustrated in this cartoon. The modest swelling of the cell of 5 to 7% of the isotonic volume, which mimics the size these cells swell when they absorb Na^+-nutrients, results in an alkalinization of pH_i. This change in pH_i is due to activation of the NHE-1 isoform of the Na^+-H^+ exchanger. A G protein mediates the activation of NHE-1. The required K^+ loss for RVD occurs via a CTX-sensitive K^+ channel. The activation of this channel absolutely requires alkalinization of pH_i. RVD proceeds because the activation of NHE-1 alkalinizes the pH_i, which, in turn, allows for activation of CTX- sensitive K^+ channels.


Na+
Solute
Ca2+
pHi
5-7%
Hypotonic
Dilution
β
γ
α
H+
Na+
H+
NHE-1
Na+
Na+
Solute
Ca2+
K+
CTXsens
[Ca2+]i
pHi
β
γ
α
H+
Na+
K+, Cl-, H20
H+
Na+

Insight into the role of GTP-binding proteins (G proteins) in activating NHE-1 for RVD after modest `physiological' swelling was obtained from experiments where these cells, when loaded by electroporation with a G protein antagonist (GDPßS), failed to alkalinize their pH_i or regulate volume reduction after the modest volume increase. Evidence that a G protein mediated the activation of NHE-1 was obtained from experiments loading these cells with a non-hydrolysable, G protein agonist (DMNPE- GTPγS). Suspended in isotonic medium and following photolysis, the cells alkalinized their pH_i. Because this change in pH_i was prevented by methyl-isobutyl-amiloride,or in the absence of MIA ,by the G protein antagonist GDPßS, I conclude that a G protein activates NHE-1 during the modest cell swelling. This conclusion was strengthened by experiments measuring the rates of pH_i recovery from an acid load in isotonic medium of cells loaded with the G protein agonist, GTPγS. The MIA-sensitive rate of pH_i recovery had this hierarchy:

$$\text{Isotonic} + \text{GTP } \gamma S = 5\% \text{ Hypotonic} > \text{Isotonic} \qquad \text{eq. 2}$$

Furthermore, intact microfilaments were required to transduce the activation of the G protein trimer since cytochalasin D was shown to both inhibit the alkalinization of pH_i that the modest swelling stimulated as well as inhibit the increased rate of MIA-sensitive pH_i recovery in 5% hypotonic medium.

Therefore, I conclude that with modest presumably `physiological' swelling of the villus cells, a G protein is activated. The effector of this G protein is NHE-1 (Figure 1).

It is not the increased Na+ influx from the activated NHE-1 which is essential for the subsequent volume regulation but, rather, the alkalinization of pH_i which is absolutely required for this RVD. We know this because when RVD was prevented by either MIA or the G protein antagonist, mimicking the extent of alkalinization (that would otherwise occur) by NH_4Cl addition, caused RVD to proceed to completion. The converse, acidifying these cells, caused the RVD to remain inhibited. Thus, the small alkalinization of pH_i which modest villus cell swelling generates is a required determinant of the ion channels activated for the subsequent volume regulation. It is the K^+ channel which is activated for this RVD which absolutely requires an alkalinization of pH_i for its activation (Fig 1). This K^+ channel is blocked by charybdotoxin (CTX), a well characterized inhibitor of Ca^{2+} activated K^+ (maxi-K^+) channels. The RVD after `modest' swelling is prevented by CTX whereas RVD after extreme osmotic challenge is not affected. The increased rate of ^{86}Rb efflux caused by a 7% hypotonic dilution was attenuated an equivalent amount by CTX, Na^+ free medium or the amiloride derivative, MIA. Furthermore, charybdotoxin-sensitive volume changes were stimulated in villus cells swollen because of extreme osmotic challenge provided that the pH_i of the cells was made alkaline. The relationship between the amount a villus cell swells as a function of the sensitivity of RVD to amiloride was superimposable on the sensitivity to charybdotoxin. As other studies suggest while $[Ca^{2+}]_i$ increases occur with villus cell swelling of both `modest' and

larger amounts (Macleod et al.1992A), the current studies suggest that it is the alkalinization of pH_i which is the critical determinant of the activation for CTX-sensitive K^+ channels utilized for RVD after modest `physiological' volume increases.

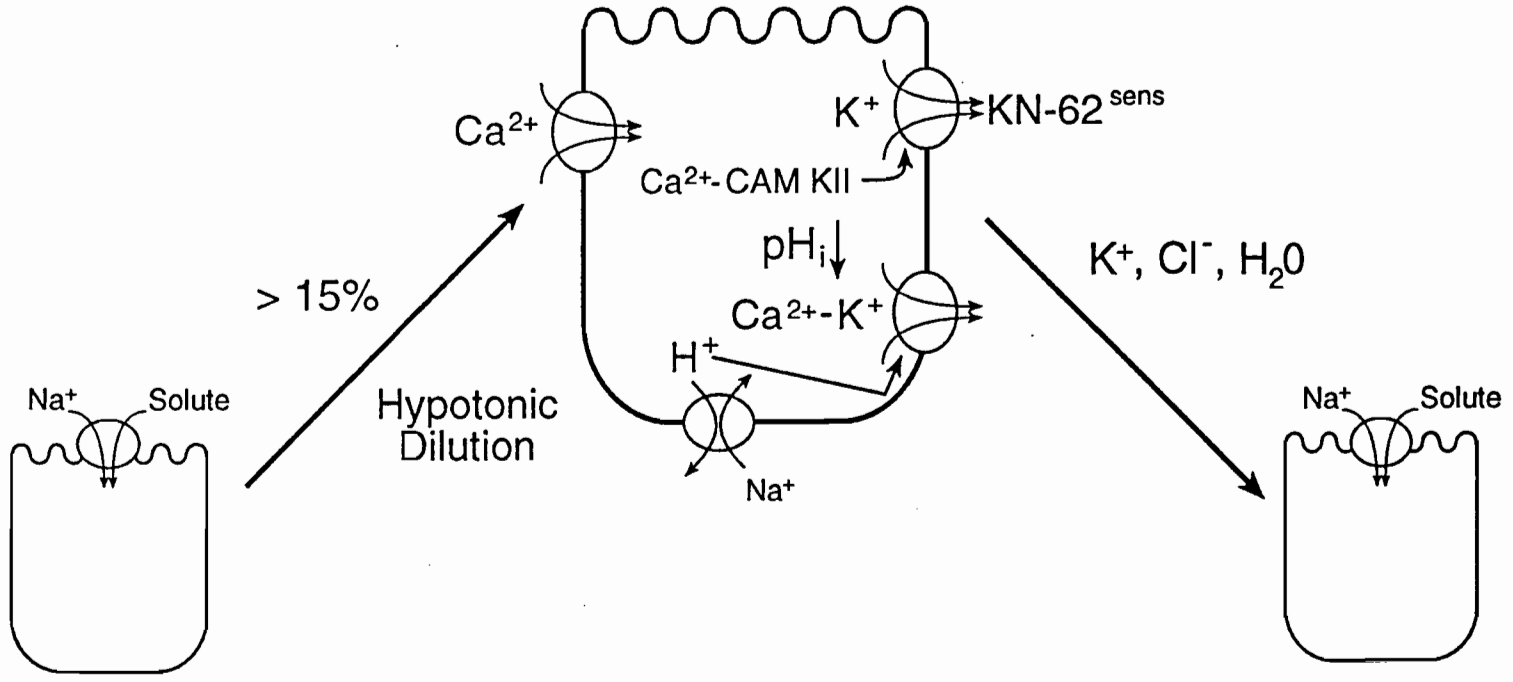
2. **Conclusion (see Fig. 1).**

I conclude that the modest, 5 to 7% volume increase, in a manner requiring intact microfilaments, activates a G protein. The effector of this activated G protein is the basolateral isoform of the Na^+/H^+ exchanger, NHE-1. The increase in pH_i which occurs because of the activated NHE-1, is an absolute requirement for activating the charybdotoxin-sensitive K^+ channel.

3. **Signal transduction of volume regulation after large volume increases**

The signalling of K^+ channel activation after large volume increases after extreme osmotic challenge (Fig.2) contrasts substantially with the activation of CTX-sensitive K^+ channels for RVD after modest volume increase. With large volume increases the pH_i does not alkalinize, it acidifies, in part due to an inhibition of NHE. The subsequent RVD is insensitive to CTX but is blocked by an inhibitor of Ca^{2+}-calmodulin kinase-II (KN-62). Because this inhibition was by-passed by the cation ionophore gramicidin, only the K^+ channel and not the Cl channel was influenced by the inhibitor of Ca^{2+}-calmodulin kinase-II, suggesting this CTX-insensitive K^+ channel was activated by phosphorylation. Consistent with this interpretation, inhibitors of phosphoprotein phosphatases accelerated volume

Figure Two: This cartoon illustrates the findings of experiments described in Chapters Two and Five. With greater volume increases, generated by extreme hypotonic dilutions, the pH_i of these cells becomes acidic, in part because of inhibited NHE-1. The RVD occurs because of K^+ loss via a CTX-insensitive channel. This K^+ channel is blocked by an inhibitor of Ca^{2+} calmodulin/kinase II. Inhibitors of phosphoprotein phosphatases accelerate volume regulation, suggesting that the CTX-insensitive K^+ channel is activated by phosphorylation. The inhibitor of Ca^{2+} calmodulin/kinase II had no effect on RVD after modest volume increases of 5 to 7%, which mimic the size these cells swell when they absorb Na^+-nutrients. While the same osmolyte is lost (K^+) for RVD when the cell swells a `physiological' amount or becomes larger with greater osmotic challenge, different K^+ channels are activated. The signals transducing the activation of these K^+ channels rely on how the pH_i of the cell changes with swelling. Thus, the extent of cell swelling is a determinant of the class of K^+ channel activated for volume regulation.


Ca2+
K+
KN-62 sens
Ca2+-CAM KII
pHi↓
Ca2+-K+
H+
Na+
Na+
Solute
> 15%
Hypotonic
Dilution
K+, Cl-, H20
Na+
Solute

reduction of these cells after large volume increases. In contrast with these findings, KN-62 had no effect on the RVD after modest `physiological', volume increases.

4. **Conclusion (see Figs 1 & 2).**

The experiments described in this thesis clearly demonstrate that the activation of K^+ channels required for RVD relies on the change in pH_i that occurs with cell swelling. The change in pH_i, alkalinization with modest swelling, acidification with larger volume increase, is strictly dictated by the extent of cell swelling. As such, the extent of cell swelling is a determinant of the signal transduction of K^+ channels responsible for volume regulation.

5. **Is there a "physiological" size associated with Na^+-solute absorption?**

The G-protein mediated activation of NHE-1 which results in K^+ loss via CTX-sensitive K^+ channels occurs only with modest, 5 to 7% volume increases. Yet this volume increase corresponds to the size these cells swell because of Na^+-solute absorption (MacLeod & Hamilton 1991; MacLeod et al. 1992 A,B). Therefore, I conclude because of the unique signalling of RVD with the modest volume increase, that there is a `physiological' volume increase which is distinct to Na^+-absorbing epithelial cells found on the villus compartment of the jejunum. It is notable that all other studies of RVD in Na^+-absorbing epithelia have relied on generating large volume increases after extreme (30 to 50% hypotonic dilutions) osmotic challenges (reviewed by Foskett, 1994). In this regard, it has been

reported that lymphocytes do not exhibit swelling activated Ca^{2+} influx after 10% hypotonic dilution, and only ~5% show $[Ca^{2+}]i$ transients after 20% hypotonic dilution, while the majority of these symmetrical cells generated swelling activated Ca^{2+} influx in response to 40% hypotonic dilution (Ross and Cahalan, 1995). It may be that the differences in signalling RVD described in this thesis (`physiological' size vs. extreme osmotic challenge sizes) is a property of Na^+-absorbing epithelia. It would be of interest to know if other epithelia which have apical Na^+-solute cotransporters exhibit the same volume determinants of pH_i homeostasis and K^+ channel activation for RVD as the villus enterocytes.

6. **Future Prospects.**

At least two experimental programs may be imagined from the work presented in this thesis. The first program would seek to answer the following question:

6a. <u>What is the molecular identity of the volume-sensitive K^+ channel which requires Ca^{2+}/calmodulin kinase-II for activation</u>?

The experiments described in Chapter Five suggest the K^+ channel activated because of volume increases > 7% is phosphorylated. Further research might begin by assessing autoradiographs of membranes prepared from villus cells, loaded with ^{32}P and then hypotonically diluted so the cells swelled >7%. These gels would be compared with comparably treated epithelial cells isolated from the crypt compartment. This is because it has been shown that crypt cells lack volume-sensitive K^+ channels (MacLeod et al. 1992C) but, like the villus cells, possess the

CTX-sensitive K^+ channel (MacLeod et al. 1994A). We would expect phosphorylated bands present in the villus preparations but absent in membranes prepared from crypt cells. Given the rapid advances in identifying K^+ channel genes (Deutsch and Chen, 1993; Lewis and Cahalan, 1995)) it should be possible to identify by molecular weight the volume-sensitive K^+ channel, assuming, of course, this channel is directly phosphorylated by Ca^{2+}/calmodulin kinase-II. The next step would be to transfect a crypt-like cell line which lacks a volume-sensitive K^+ channel (i.e. IEC-6) with the K^+ channel identified from the comparison of crypt with villus membrane preparations. Subsequently, structure-function analysis would be performed to determine how this K^+ channel is activated by phosphorylation because of large volume increases.

A second program could address the following:

6b **<u>Identifying the G protein responsible for activating NHE-1 with modest (5 to 7%), `physiological' volume increases</u>**.

Chapter 3 described experiments demonstrating the activation of NHE-1 for RVD is mediated by a G protein. This G protein was shown to be pertussis toxin insensitive as well as protein kinase C insensitive. Since it has been established that the Gα12 subunit family is insensitive to pertussis toxin and constitutively active Gα13 will stimulate NHE in protein kinase C independent manner (Dhanasekaran et al., 1994), one approach to answering the above question would be to first establish the villus cells in primary culture and then determine whether

Gα13 is responsible for NHE-1 activation. If these cells manifested the same pH_i and volume responses of the freshly isolated cells, then villus cells in primary culture could be treated with antisense oligonucleotides of Gα13. Assuming that the turnover of Gα13 protein is within the same period of time these cells could be maintained to show volume and pH_i responses comparable to freshly isolated cells, then the cultured villus cells treated with antisense oligonucleotides would show no change in pH_i or volume regulation after the modest hypotonic dilution. This antisense oligonucleotide approach has been successfully used in assessing developmental changes of CFTR in d18 fetal distal pneumocytes (MacLeod et al., 1994B) and cation channels in non-epithelial cells (Duncan et al., 1996). If antisense treatment prevented the pH_i and volume changes, which would implicate Gα13 in these events, Gα13 would then be isolated from the villus cells using standard techniques (Higashjuma et al. 1987A).

6c: <u>How is volume sensed</u>?

In Chapter 3 it was shown that an intact microfilament network was required to activate the G protein prior to it activating its effector, NHE-1. It is well known that the activity of G protein trimers are regulated in a cyclic manner by various turn-on and turn-off reactions, and that this cycle usually begins after an agonist binds its receptor to generate a conformational change in the receptor (Gilman, 1987; Brown and Birmbaumer, 1990). Some G proteins are sensitive to halides; both the `on' and `off' reactions will change as a function of $[Cl^-]_i$ (Higashijima et al. 1987 B,C; Nakajima et al. 1992). Further experiments could assess whether Gα13

is halide sensitive, and if a change in $[Cl^-]_i$ with `modest' , physiological volume increase promotes G protein trimer dissociation. Alternatively, if the G protein responsible for activating NHE-1 was found to be linked to actin or an actin binding protein, the hypothesis to test would be that the torsional change associated with the modest, 5 to 7% volume increase causes the G protein trimer's dissociation. Such experiments would focus on whether it is the physical distortion accompanying modest swelling which activates the G protein or whether a change in $[Cl^-]_i$ causes a halide-sensitive G protein to dissociate. The unique characteristics of guinea pig jejunal villus epithelial cells may be used to gain considerable insight into the nature of the volume `sensor'.

7. Concluding Remark

The activation of the K^+ channel required for RVD relies on the pH_i change that accompanies cell swelling. Modest `physiological' volume increases result in a G protein activating basolateral NHE-1 which alkalinizes the cell. Activation of the CTX-sensitive K^+ channel, which is utilized for RVD, absolutely requires alkalinization of pH_i for activation. In contrast, extreme osmotic challenge, resulting in much greater swelling, causes the pH_i to acidify. The K^+ channel activated is insensitive to CTX but has characteristics consistent with its activation requiring phosphorylation. Because the change in pH_i is determined by the extent of villus cell swelling, it is concluded that the extent of cell swelling is a signalling determinant of the class of K^+ channel required for volume regulation.

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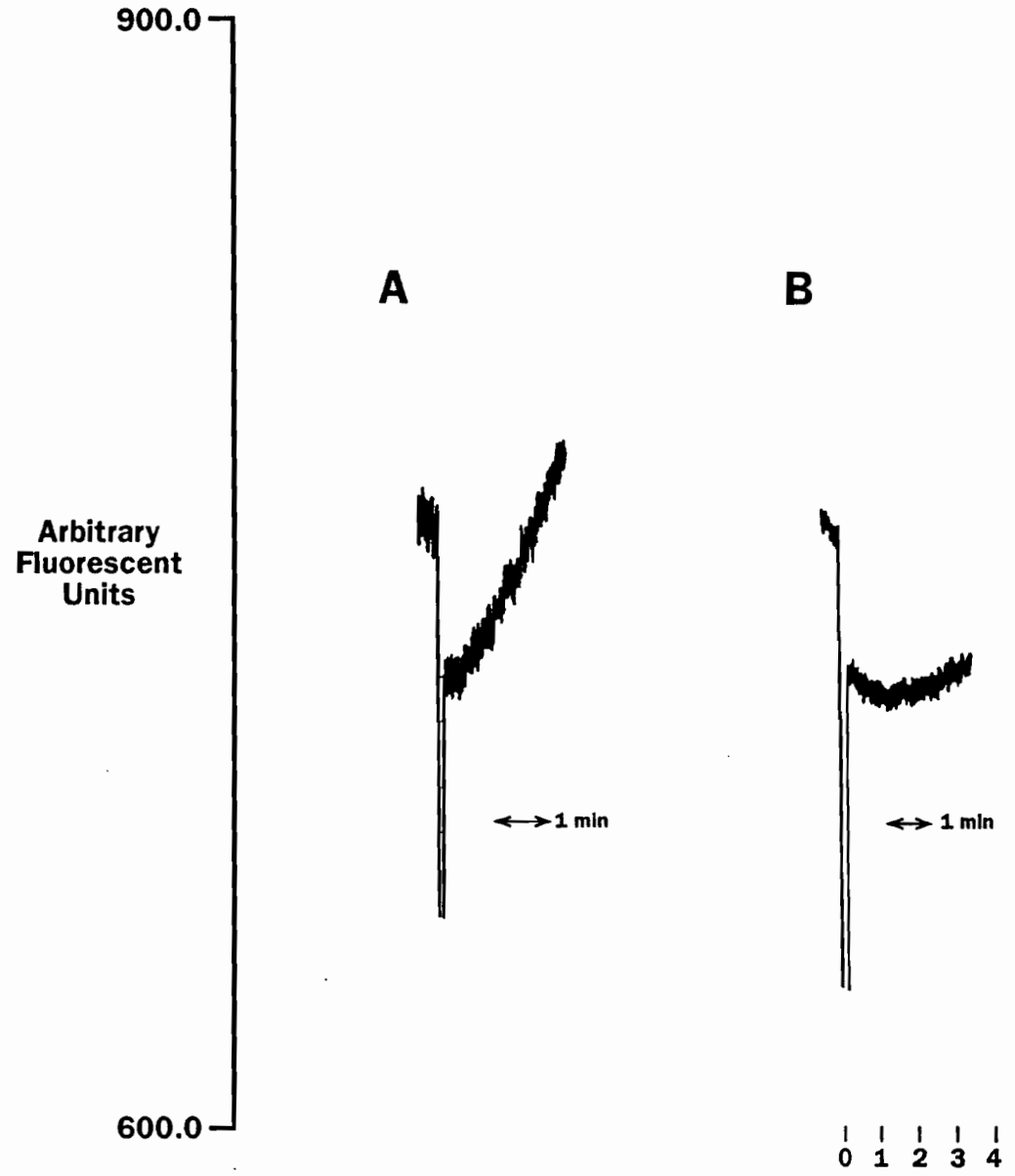
Annex

Correcting for Dilutional Artifacts

The fluorescent intensity of the pH_i-sensitive dye loaded into the villus cells is exquisitely sensitive to the density of cells in suspension. Changing cell density by hypotonic dilution must be corrected by an appropriate isotonic dilution.

Illustrated in Figure 1 is the change in fluorescent intensity of cells after a 5% hypotonic dilution (A) or 100 µl of distilled water to 2 mls of cells, compared with a 5% isotonic dilution (B). The break in the tracing is from opening and closing the spectrofluorometer's lid. Because the isotonic dilution caused a decline of 38 arbitrary fluorescent units, 38 units were added to the units after the 5% dilution. This correction had no effect on $\triangle pH_i$/3min or any <u>net</u> pH_i changes.

Figure 1 (Annex) : Effect of Isotonic Dilution



Contributions to Original Knowledge*

This section is a mandatory requirement of Ph.D. theses submitted to the Faculty of Graduate Studies and Research, McGill University, Montreal, Canada.

The following novel findings are described in this thesis.

1. A new pathway for Regulatory Volume Decrease (RVD) in Na^+-absorbing intestinal epithelial cells has been characterized.

2. Modest volume increases, which duplicate those experienced by intestinal villus cells during Na^+-solute absorption, activate the NHE-1 isoform of the Na^+/H^+ exchanger. Larger volume increases caused by greater osmotic challenge inhibit NHE-1.

3. The subsequent RVD after modest volume increases of 5 to 7% absolutely require activated NHE-1 whereas RVD after larger volume increases does not.

4. It is the alkalinization of pH_i, not the increased Na^+ influx which is required for the RVD after modest volume increases.

5. A charybdotoxin (CTX)-sensitive, Ca^{2+} activated K^+ channel is the determinant of K^+ loss and consequent RVD after modest volume increases. This CTX-sensitive K^+ channel requires the alkalinization of pH_i for its activation. The K^+ channel activated for RVD after greater volume increases, which cause the pH_i to acidify, is CTX-insensitive.

6. A G-protein, not intracellular acidification, mediates the activation of NHE-1 during modest volume increases. This G-protein mediated activation requires intact microfilaments.

7. Ca^{2+}-calmodulin kinase II mediates the activation of the CTX-insensitive K^+ channel required for RVD after greater volume increases.

8. The size these epithelial cells swell determines whether NHE-1 is activated or inhibited. The change in pH_i is a determinant of the K^+ channel activated for the subsequent volume regulation.

symmetrical cells that NHE-1 accumulates in sites which are also sites of accumulation of vinculin, tailin and F-actin (15). Some integrins will serve as anchors for cytoskeletal structures composed of tailin and ∝-actin (32). While no direct evidence links NHE-1 with a cytoskeletal element, in renal epithelia the Na^+/K^+ ATPase is linked to actin by ankyrin and spectrin (29). Furthermore, Na^+ channels in A6 epithelial cells will increase their open probability by cytochalasin D treatment and immunochemical evidence has shown these channels localized with actin, spectrin and ankyrin in the microvilli of these cells (33). Because DHCB prevented the swelling induced ΔpH_i, our results suggest that an intact actin cytoskeleton is necessary to transduce the G protein-mediated activation of NHE resulting from the modest volume increases. Actin microfilaments could be linked to a putative receptor/volume sensor which is coupled to a G protein; alternatively, some microfilaments might be directly coupled to a trimeric G protein or indirectly linked to NHE. Our experiments do not allow us to discriminate between these models, but the latter is unlikely because acid-loaded enterocytes still manifested MIA-sensitive pH_i recovery in the presence of DHCB (Fig 5C). Therefore, we speculate that if a G protein complex were directly coupled to actin microfilaments, mechanical stretching associated with a physiological volume increase of 5 to 7% might directly activate the G-protein via these microfilaments, in a manner analgous to the conformational change of a receptor caused by agonist binding leading to G protein activation (16,17). In these epithelial cells, the volume sensor would be a microfilament-linked G protein. There is evidence that G proteins are coupled to some cytoskeletal elements. In symmetrical cells β subunits of G proteins co-