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Rapid Report

Regulatory volume decrease in small intestinal crypts is inhibited by K^+ and Cl^- channel blockers

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Total crypt volume has been estimated by analysis of photographic images of intact viable crypts isolated from guinea-pig small intestine. Exposing these crypts to a hypotonic medium, led to transient swelling followed by regulatory volume decrease (RVD) in 12–20 min. RVD was blocked by inhibitors of K^+ and Cl^- conductance, suggesting that it occurs by activation of K^+ and Cl^- permeability pathways and loss of these ions.

Many animal cells have been demonstrated to alter their cell osmolyte content during exposure to anisotonic media by the transient activation of transport pathways leading to associated water movement. In response to swelling in a hypotonic environment cells effect the passive loss of organic osmolytes and/or KCl in a process termed regulatory volume decrease (RVD, see Ref. 10 for review). Most studies of cell volume regulation have so far been conducted on non epithelial cells, though it might be assumed that cells in transporting epithelia will be exposed more readily to both intra- and extracellular anisotonicity due to the nature of their transport activity. In particular in the small intestine it is known that changes in the rate of Na^+/nutrient cotransport induce changes in K^+ and Cl^- permeability which were assumed to maintain constant cellular volume [3,6,9]. This assumption has recently been confirmed by direct measurements of cellular volume carried out with guinea-pig intestinal villus epithelial cells [13]. Direct measurements of RVD in response to hypotonic shock in the same cells have also demonstrated that villus enterocytes regulate their volume through KCl efflux via separate K^+ and Cl^- permeability pathways [14].

Little is known about the transport properties of crypt cells and no information is available on whether they possess cell volume regulatory mechanisms. The crypts have long been postulated to be the site of small intestinal secretion, and some direct evidence for this contention is starting to emerge [19]. It could reasonably be assumed that they would need homeostatic regulatory mechanisms to maintain their volume during secretion. We have therefore used isolated intact crypts and image analysis of light microscopy pictures to infer changes in cell volume. We demonstrate that intact crypts isolated from the guinea-pig small intestine regulate their volume upon exposure to hypotonic media, and that RVD appears dependent on the activation of both K^+ and Cl^- permeability pathways.

The isolation method used here involves Ca^{2+} chelation and vibration, and yielded intact sheets of villi and whole crypt sequentially. This could be confirmed by observation with a microscope but also by following the specific activity of crypt and villus marker enzymes. Fig. 1 shows the activity of alkaline phosphatase, a marker for mature villus enterocytes, and thymidine kinase, a marker for proliferative crypt cells in the different fractions collected. Within 4 min 85% of the total alkaline phosphatase specific activity isolated was recovered in the medium bathing the everted intestine. During the same period 35% of the total thymidine kinase was shed. By contrast the fractions collected between minutes 16 and 22 had 0.01% of the total alkaline phosphatase and 25% of thymidine kinase. Intermediate values were observed between these times, suggesting that villus and crypts had been isolated sequentially. In addition microscopic observation of the fractions also revealed that early fractions were enriched in caps of villus epithelial cells while the last fraction was greatly enriched in morphologically distinguishable, near-cylindrical crypts. In the last, a luminal virtual space could often be discerned. Trypan blue

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was excluded initially from all morphologically intact crypts that we could normally identify as 'healthy' by their birefrigence under phase-contrast microscopy. A similar result was obtained with propidium iodide, which stained the nuclei of dead cells red when viewed under fluorescent confocal microscopy. Crypts remained viable for at least 60 min at 22°C and for up to 8 h when kept in Dulbecco's modified Eagle's medium (DMEM) on ice. In addition we have recently done membrane potential measurements in the same preparations and obtained results suggesting that cellular integrity was being maintained under these conditions [19].

Crypts maintained in isotonic solution showed a tendency to shrink slightly during incubation. This is shown in Fig. 2 where the estimated volume, relative to initial volume, is plotted as a function of time after simply renewing the isotonic solution contained in the perfusion chamber. The osmolality of the bathing solution was reduced at the time marked zero in the abscissa, without altering the ionic composition, by removing 70 mM mannitol. This led to a rapid increase

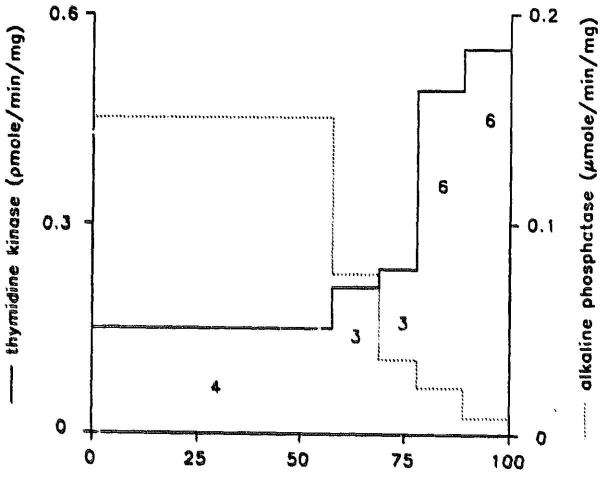

Fig. 1. Histogram showing thymidine kinase and alkaline phosphatase specific activities in epithelial fractions isolated from guinea-pig small intestine against % of total protein isolated in each fraction. Times of successive fractions are given in min. Ileal epithelium was isolated as described in Refs. 1 and 15. Guinea-pigs weighing 250–350 g were starved for 12 h and killed by cervical dislocation. A 25–30 cm length of ileum was excised and washed twice before being everted and ligated to a perspex rod. The preparation was then vibrated at 50 Hz in Ca^{2+}-free solution at 4°C; (composition in mM, 30 Na_2 EDTA; 5 KCl; 60 HCl; 52 NaCl; 2 DTT; 10 Hepes (pH 7.1)) for timed intervals over 22 min. Successive fractions were collected and washed material was resuspended in DMEM containing 0.1% bovine serum albumin. The crypts were kept on ice and stayed viable for up to 8 h. Viability was determined at room temperature by testing for the exclusion of 0.03% Trypan blue or 5 mg/ml propidium iodide. Alkaline phosphatase was assayed using *p*-nitrophenyl phosphate as substrate [20]. The assay for thymidine kinase followed the method in Ref. 11 but isolating phosphorylated thymidine with a cationic Dowex resin [8]. Protein was assayed with bicinchoninic acid using a commercial kit (Pierce).

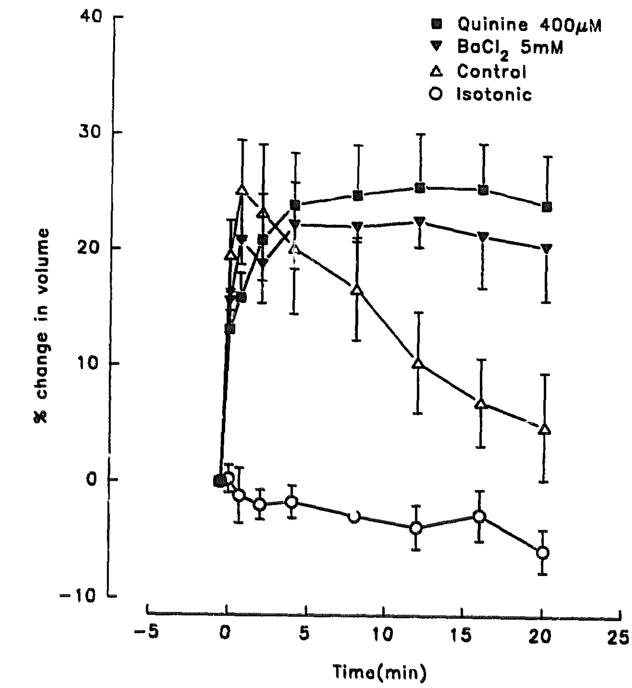

Fig. 2. Relative changes in isolated crypt volume in response to changes in osmolality of the bathing medium. Zero time is defined as the time of solution change. The bathing solution was replaced with isotonic solution or with hypotonic solution with or without the indicated blockers. The effects of the addition of quinine and Ba^{2+} in isotonicity are included in the mean values of the isotonic controls. Values are means ± S.E. of six experiments. Cell volume regulation was observed with an inverted microscope (total magnification 320×) in a perspex chamber (total volume 1 ml) whose bottom was made out of a glass coverslip. Adherence of the crypts was achieved by precoating the glass surface with 0.1% polyethyleneimine. An aliquot of crypts suspended in an isotonic (300 mosM) solution (containing, in mM, 105 NaCl, 2.5 KCl, 0.5 $MgCl_2$, 1.2 $CaCl_2$ and 70 mannitol, 10 Hepes (pH 7.2)) was added to the chamber and left to equilibrate for 15 min before washing with 10 ml of isotonic solution. Solution replacement was made by perfusion of 10 ml of the desired solution under continuous suction. A cylindrically-shaped individual whole crypt was selected and photographs taken at timed intervals. Photographs of the crypts were analysed for total area, mean height (h) and mean width ($2r$) with the aid of a Quantimet 520 image analysis system. Volume was estimated assuming a cylindrical shape, as $\pi \cdot h \cdot r^2$, and normalised to the point previous to solution changes. The hypotonic solution had a tonicity of 225 mosM and was prepared as the isotonic solution but omitting mannitol.

in apparent volume of about 25% within the first minute after solution change. After the initial swelling, crypts slowly regained their volume over a period of about 20 min.

In order to test whether K^+ or Cl^- permeability pathways were involved in crypt RVD, the effect of K^+ and Cl^- channel blockers was tested. Fig. 2 shows that the K^+ channel blockers quinine or $BaCl_2$ added at the time of the switch from iso- to hypotonicity did not markedly affect the pate or the extent of the osmotic increase in volume. In contrast, the blockers did abolish RVD, the crypts remaining swollen for the duration of the experiment. The addition of blockers in the

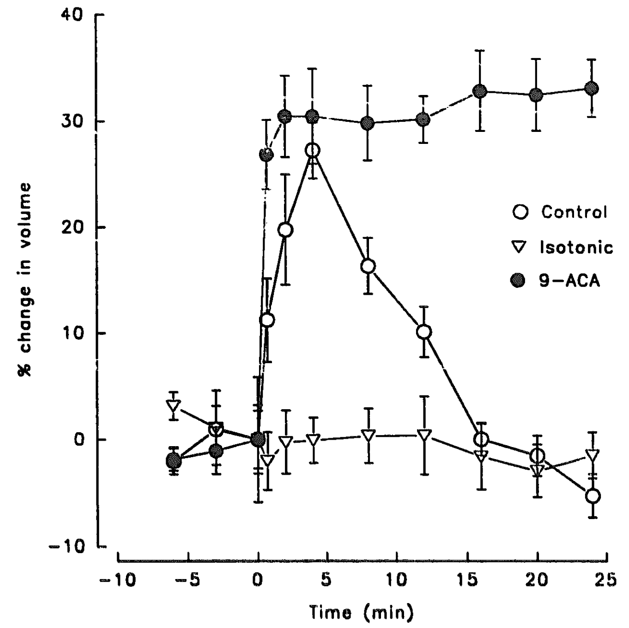

Fig. 3. Relative changes in crypt volume before and after the substitution of the isotonic bathing medium with hypotonic solution with or without the inhibitors indicated. Results are means ± S.E. of six experiments.

absence of hypotonic challenge did not produce any significant effect on crypt volume and the effect of the blockers was reversible as the crypts recovered their volume if perfused again with isotonic solution (results not shown).

Cl^- permeability pathways are also known to play a role in RVD in a variety of other cell types. In order to test for the possibility of a similar involvement in the crypt RVD the effect of the Cl^- channel blockers 9-anthracene-carboxylic acid (9-ACA) and 4,4′-diisothiocyanostilbenedisulphonic acid (DIDS) was tested. Fig. 3 shows the effect of hypotonic challenge in the absence of inhibitors and in the presence of 100 μM 9-ACA. Maximal swelling was observed within 4 min and RVD was complete in 16–20 min in this series of experiments. When the switch from iso- to hypotonicity was done in the presence of the blocker a fast increase in volume was observed, but unlike the control situation the volume remained elevated for the duration of the experiment. Crypts maintained in isotonic medium did not change their volume markedly and 9-ACA had no effect under these conditions. A series of separate experiments was conducted to test for the effect of DIDS on the RVD process. Hypotonic challenge produced an increase in volume by 19 ± 2 and $15.5 \pm 1\%$ in the absence and presence of 100 μM DIDS, respectively; the corresponding values at the completion of RVD, which occurred after 12 min in these experiments, were -0.6 ± 1 and $10.2 \pm 2\%$ (means $\pm$ standard errors of four experiments).

The aim of the present series of experiments was to ascertain whether it is possible to use intact crypts in volume regulation studies, to see whether they were capable of regulating their volume in response to osmotically-induced water movement and to make initial observations as to the possible mechanisms of volume regulation. Studies of volume regulation in epithelial cells have often been carried out by electronic sizing of dissociated cells in suspension [14,16]. This approach is not possible with the intact epithelium and we therefore resorted to approximate measurements of whole-crypt size by analysis of timed photographic images. The method, which involves the assumption that the crypt shape can be approximated to that of a cylinder, appears to give a reasonable measure of cell volume as the initial response to hypotonicity was similar to that expected from a perfect osmometer. A similar approach to measure volume regulation of individual cells has been used before by others [4,17,18].

In our experiments, after the initial swelling in hypotonic medium the cells recovered their volume in a process which is complete within 12–20 min, with 50% RVD completed within 2–8 min of exposure to hypotonic medium. It is interesting to note that the process of RVD is considerably slower in the intact crypt epithelium than is generally reported for experiments with isolated single cells: such as the cultured Intestine 407 cell line [16] and isolated jejunal villus enterocytes [14]; whether these time-dependent differences are due to surface area to volume ratio effects or specific differences in cellular physiology is not clear.

RVD in cells exposed to hypotonic media has been shown in other cells to be effected by the loss of organic osmolytes and/or KCl down an electrochemical potential gradient upon the activation of normally quiescent regulatory pathways [10]. Three different mechanisms have been proposed to be involved in mediating net KCl efflux during RVD [10]: activation of electroconductive channels selective to both anions ' K^+; electroneutral cotransport and by functionally ıpled H^+/K^+ and Cl^-/HCO_3^- exchange. Our results are consistent with the participation of independent K^+ and Cl^- channels in the RVD process of small intestinal crypts as it is possible to interfere with volume regulation with specific blockers of these permeability pathways.

Ba^{2+} and quinine which are both known blockers of K^+ channels abolish RVD in isolated villus enterocytes and in the Intestine 407 cell line [14,16] and, used at concentrations similar to those employed in previous work, abolish RVD in crypts from guinea pig small intestine. The evidence for the involvement of Cl^- permeability pathways comes from the use of 9-anthracene carboxylic acid, a Cl^- channel blocker, which also abolishes RVD of small intestinal crypts. On the basis of this pharmacological evidence it would appear that crypt RVD occurs through K^+ and Cl^- release via separate conductive membrane pathways.

DIDS, a stilbene derivative, also inhibits RVD in intestinal crypts. This type of blocker is primarily an inhibitor of Cl^-/HCO_3 exchange but it has also been reported to block intestinal Cl^- channels [2,5].

Previous studies of volume regulation in intestinal cells have not elucidated which membrane domain, apical or basolateral, harbours the K^+ and Cl^- conductive pathways involved in RVD. In enterocytes from Necturus small intestine electrophysiological recordings suggest that a Ba^{2+}-sensitive, swelling-activated conductance is basolateral [12] and a swelling activated Cl^- conductance is located in the apical membrane [7]. Although it is not possible to ascribe a site for the conductive pathways postulated here, it is tempting to speculate that they are basolateral. Such a location would be consistent with the virtually instantaneous effect of the blockers, as the lumen of the crypts should not be readily accessible to the bathing solution. Another question which cannot be answered with the present results is whether cell swelling activates previously dormant conductances. Our electrophysiological recordings have revealed the presence of a Ba^{2+}- and quinine-sensitive basolateral K^+ conductance in unstimulated guinea-pig small intestinal crypts [19]. It would then be possible to envisage that Cl^- permeability is the rate-limiting step in RVD with K^+ efflux taking place through a tonically active K^+ conductance. This problem, and the question of the possible mechanism linking the increase it, volume with the changes in permeability in the crypts, might be solved by direct measurement of the conductance changes by electrophysiological means.

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