Abstract

A spatial segregation of ion transport processes between crypt and surface epithelial cells is well-accepted and integrated into physiological and pathophysiological paradigms of small and large intestinal function: Absorptive processes are believed to be located in surface (and villous) cells, whereas secretory processes are believed to be present in crypt cells. Validation of this model requires direct determination of fluid movement in intestinal crypts. This study describes the adaptation of techniques from renal tubule microperfusion to hand-dissect and perfuse single, isolated crypts from rat distal colon to measure directly fluid movement. Morphologic analyses of the isolated crypt preparation revealed no extracellular cellular elements derived from the lamina propria, including myofibroblasts. In the basal state, crypts exhibited net fluid absorption (mean net fluid movement = 0.34±0.01 nl·mm⁻¹·min⁻¹), which was Na⁺ and partially HCO₃⁻ dependent. Addition of 1 mM dibutyryl-cyclic AMP, 60 nM vasoactive intestinal peptide, or 0.1 mM acetylcholine to the bath (serosal) solution reversibly induced net fluid secretion (net fluid movement ∼ -0.35±0.01 nl·mm⁻¹·min⁻¹). These observations permit speculation that absorption is a constitutive transport function in crypt cells and that secretion by crypt cells is regulated by one or more neurohumoral agonists that are released in situ from lamina propria cells. The functional, intact polarized crypt described here that both absorbs and secretes will permit future studies that dissect the mechanisms that govern fluid and electrolyte movement in the colonic crypt. (J. Clin. Invest. 1995. 96:2373–2379.)

Keywords: fluid secretion • myofibroblasts • cyclic AMP • intestine • crypt

Introduction

Studies of intestinal electrolyte transport performed >25 yr ago established that net fluid and electrolyte movement in the small and large intestine is determined by both absorptive and secretory processes (1, 2). For the past two decades, the accepted paradigm has been that, within a given intestinal segment, absorptive and secretory processes are spatially segregated along the axis from the superficial colonic surface epithelium (or villous cells in the small intestine) to the gland like crypt: Absorptive processes are present exclusively in the surface epithelial cells in the colon, whereas secretory processes are located exclusively in crypt cells (3, 4). However, recent studies in rat colon (5) and jejunum (6) suggest that secretory mechanisms also may be present in surface and villous cells. To date, there has been no direct evidence that absorptive processes are located in crypt epithelium, although it has been suggested, based on theoretical considerations and indirect observations, that crypts absorb fluid (7, 8).

Direct functional studies of crypt epithelium have been hindered by the absence of methods to study fluid and electrolyte movement in polarized crypt cells from native intestine. The T₄₈ cell line, derived from a human colonic carcinoma, is well-maintained as a monolayer and has been used to great advantage as a model of crypt cell function. T₄₈ cells in their basal state manifest 0 net Na⁺ and Cl⁻ transport, but several agonists and intracellular messengers induce active Cl⁻ secretion (9). Active Na⁺ absorption has not been demonstrated in T₄₈ cells under any experimental conditions. However, as these cells are derived from a carcinoma, the transport processes present in T₄₈ cells may not reflect truly the properties of normal crypt epithelial cells.

A few investigators have used isolated crypts and/or relatively enriched crypt cell preparations to study one or more aspects of crypt cell function, including electrophysiology, cytosolic receptor binding, intracellular pH, cell volume, and ion uptake (10–15). In most of these studies, crypt cell polarity has not been preserved, and in none has fluid and/or ion transport been determined in an intact crypt epithelium. Because epithelial cells are polarized, a thorough understanding of their transport physiology requires that epithelial polarity and intercellular junctions be maintained.

A direct approach to establish whether secretion is indeed an exclusive function of crypt epithelial cells would require study of the rate of fluid movement (Jₑ) in intact crypt epithelium independent from surface cells. In the present study, colonic crypts were isolated and perfused simultaneously and independently on their luminal and serosal surfaces. These microperfusion techniques have been used previously in the study of isolated nephron segments (16) and gastric glands (17) and were adapted for the direct determination of Jₑ in isolated colonic crypts. Contrary to the spatial-distribution model, which predicts that crypts only secrete, we found that the isolated colonic crypt absorbs fluid in the basal state. Moreover, addition of agonists that induce active Cl⁻ secretion in native colon and T₄₈ cells, i.e., dibutyryl-cAMP (DBcAMP), vasoactive intesti-

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1. Abbreviations used in this paper: ACh, acetylcholine; DBcAMP, dibutyryl cAMP; Jₑ, net fluid movement; NHE, Na–H exchange; VIP, vasoactive intestinal peptide.
nal peptide (VIP), or acetylcholine (ACh), reversibly stimulated fluid secretion in the colonic crypt. Based on these results, the existing model of the spatial distribution of absorptive and secretory processes between colonic surface and crypt cells must be modified; the present work suggests that absorptive processes are constitutively present in crypt cells but that multiple neurohumoral agonists released from one or more lamina propria cells, especially the myofibroblasts, regulate secretion.

Methods

Luminal perfusion of colonic crypts and determination of J,
Nonfasting male Sprague-Dawley rats weighing 100–150 g were killed; the colons were removed, and single, hand-dissected colonic crypts were perfused using the same approach used in perfusion studies of renal tubules (16, 17). The crypts were mounted in a superfusing 0.25-ml chamber on the stage of an inverted microscope (Carl Zeiss, Thornwood, NY). An assembly of concentric glass micropipettes was used to hold the blind end of the crypt; the perfusion pipette was used to puncture this blind end and introduce the perfusate containing 10 μCi/ml methoxy-[3H]inulin (New England Nuclear, Boston, MA) into the lumen of the crypt. A second set of micropipettes was used to cannulate the open end of the crypt and collect the effluent. Solutions flowed continuously through the lumen with a collection rate of ∼4 nl/min. Solutions were delivered near the tip of the perfusion pipette via a fluid exchange pipette system open to atmospheric pressure, as previously described (19, 20). Simultaneously, the bath (i.e., serosal or blood-side of the crypt) was superfused continuously in the chamber at a rate of ∼3 ml/min. Both lumen and bath solutions were maintained at the same height above the perfusion chamber and were changed without disrupting flow using multiple-inflow single-outflow pressure valves (Minuteman Controls, Wakefield, MA). The order of perturbations was randomized except for the results shown in Fig. 6. Measured aliquots of effluent were sampled with a volume-calibrated pipette. J, was calculated as previously described (19, 20) from the rate at which the effluent accumulated in the collection pipette and the concentrations of methoxy-[3H]inulin in the perfuse and effluent. [Methoxy-[3H-inulin] was determined with a liquid scintillation spectrometer (Model 6892; Tracor Analytic, Elk Grove Village, IL) with a counting accuracy of 0.5%. For triplicate samples of the collected fluid, the standard error (SE) of counts ranged from 0.10% to 0.35% of mean values. Positive J, values (nl·mm⁻¹·min⁻¹) indicate net fluid movement from lumen to bath (i.e., absorption); negative values indicate net fluid movement from bath to lumen (i.e., secretion). SE for J, ranged from 2 to 115% of the mean value, averaging ∼5%.

The bath solution was routinely assayed for methoxy-[3H]inulin, and experiments were discarded in which bath [methoxy-[3H-inulin] exceeded background. A 15-min interval followed all solution changes, after which timed collections of luminal effluent were initiated. Each data point in the figures represents the average of three ∼10-min collections of effluent. At the end of each experiment, the viability of the crypt was assessed with trypan blue, and experiments in which cells failed to exclude dye were disregarded (<15% of experiments). The HCO₃⁻-Ringer solution contained (in millimolar): 125 NaCl; 5 KCl; 1.2 CaCl₂; 1.2 MgSO₄; 2 NaH₂PO₄; 10.2 glucose; and 22 NaHCO₃. The solution was equilibrated with 5% CO₂ and adjusted to an osmolality of 300–310 mOsm/kg. pH of all solutions was 7.40 at 37°C. In the Na⁺-free solution, NaCl and NaHCO₃ were replaced with equivalent amounts of choline-Cl and choline-HCO₃, respectively, and NaNH₂PO₄ was replaced by 2 mM H₂PO₄. In the nominally CO₂/HCO₃ free solution, 32 mM Hepes replaced 22 mM NaHCO₃ and the solution was air equilibrated. The one-tailed Student's t test was used to determine statistical significance.

Morphology

Scanning electron microscopy. Hand-dissected crypts were fixed in Ringer's containing 1% glutaraldehyde at 4°C for 1 h. After being washed three times in PBS the crypts were postfixed for 1 h in 1% OsO₄ in 0.1 M sodium cacodylate buffer, then washed. The crypts were then dehydrated in graded ethanol and critical-point–dried using liquid CO₂ as the transitional fluid. Individual crypts were then mounted on suitable stubs and sputter-coated with gold. Scanning electron microscopy was performed on an electron microscope (ISI = SS = 40; Topcon Instruments, Paramus, NJ) operating at 10 kV.

Immunohistochemistry

Labeling with PR 2D3, an mAb that reacts with myofibroblasts in the pericryptal sheath (21), was performed as described previously (22). Pieces of parafomaldehyde-fixed, full-thickness tissue were rapidly thawed in PBS containing 10% DMSO and mounted in Optimal Cutting Temperature Compound® (Miles Laboratories Inc., Elkhart, IN). Cryo sections were cut at 6-μm thickness. The tissue was blocked for nonspecific fluorescent staining in PBS 1% BSA, and 1% normal goat serum for 1 h at 25°C. The primary antibody, PR 2D3, was provided as a tissue-culture supernatant and was applied undiluted to the tissue for 1 h at 25°C then washed with PBS containing 1% BSA. Secondary antibody, a goat anti–mouse Fab fragment conjugated to rhodamine (Organon-Teknika Inc., Rockville, MD) was then applied at a dilution of 1:200 for 1 h and then washed with PBS containing 1% BSA. Slides were then counterstained and edge-sealed as above. Immunofluorescence was examined by confocal microscopy (Bio-Rad Laboratories Instruments, Hercules, CA) with a rhodamine filter set. The intensity of fluorescence emission from labeled tissue was remarkably high, with virtually undetectable nonspecific background staining. No image analysis or intensification was used.

Results

Morphology and integrity of colonic crypts. Fig. 1 is a scanning electron micrograph of the basolateral surface of a hand-dissected crypt. The crypt appears to be an intact structure lacking extracellular cellular elements. Additionally, light and transmission electron microscopy revealed that the hand-dissected crypt was an intact epithelium composed primarily of two cell types, the mucus-containing goblet cells and microvacuolated cells (micrographs not shown). At no time were any lamina propria cellular elements identified.

To confirm further that hand-dissected crypts were an isolated epithelium devoid of its myofibroblast-containing pericryptal sheath, immunohistochemical studies were performed with the mAb (PR 2D3). This IgG-class antibody reacts with cells in the pericryptal sheath identified as myofibroblasts (21). Immunofluorescent activity was readily identified in the lamina propria of intact rat colon (Fig. 2 A). In contrast, PR 2D3 did not react with any cellular elements in the hand-dissected crypts (Fig. 2 C). These immunohistochemical experiments, together with the transmission and scanning electron microscopic studies, confirm that the isolated crypt preparation is devoid of subepithelial myofibroblasts.

Basal fluid movement and effect of Na⁺ removal. In the first series of experiments, the direction and rate of fluid movement in colonic crypts in the basal state was determined during perfusion of lumen and bath with identical HCO₃⁻–containing Ringer-like solutions. Fig. 3 presents the results of six separate experiments. In each case, constant rates of fluid absorption were present over three successive 10-min samples. As shown in Fig. 3, bar a, the mean basal J, was 0.27±0.03 nl·mm⁻¹·min⁻¹. These experiments demonstrate that, in the basal state, isolated
Figure 1. Scanning electron micrograph of an isolated crypt. Crypt length is typically 400 μm. The isolated, hand-dissected crypt is an intact epithelial unit without associated cellular elements. The white scale marker represents 10 μm.

Figure 2. Immunohistochemistry for localization of myofibroblasts using mAb PR 2D3. (A) Mucosal section through colonic tissue from which crypts have not been dissected. Staining is positive in a rimlike distribution adjacent to the basolateral surfaces of both crypt and surface epithelium. There was no detectable nonspecific staining. (B) Transmitted image of the hand-dissected crypt shown in C fixed in paraformaldehyde. (C) Rhodamine fluorescence image of B, stained for pericryptal myofibroblasts. No fluorescence was detectable.
perfused crypts of rat distal colon absorb fluid. This observation conflicts with the established model that fluid secretion is the exclusive function of crypt cells.

The role of Na⁺ in crypt fluid absorption was examined next. In both in vivo and in vitro studies of rat distal colon, water absorption has been found to be Na⁺ dependent (2, 23, 24). In the present studies, choline was substituted for Na⁺ in both the lumen and bath. In the absence of Na⁺, mean Jᵥ was -0.23±0.03 nl·mm⁻¹·min⁻¹, indicating that fluid absorption in the crypt was Na⁺ dependent (Fig. 3, bar b).

**Reversible induction of a secretory state by dibutyryl-cAMP.** Given that fluid absorption (not fluid secretion) was observed in the basal state, experiments were performed to establish whether isolated colonic crypts are capable of secretion. Thus, the effect on Jᵥ of DBcAMP, a permeant cAMP analogue that induces colonic Cl⁻ secretion both in native rat colon (25) and in T８４ cells (26), was determined. Fig. 4 presents the results of six experiments performed in HCO₃⁻-containing solutions in which Jᵥ was determined under control conditions, in the presence of 1 mM basolateral DBcAMP, and finally after washout of the DBcAMP. In the absence of DBcAMP, the mean Jᵥ was +0.35±0.03 nl·mm⁻¹·min⁻¹, indicating net fluid absorption (Fig. 4, bar a). Exposure to DBcAMP induced net fluid secretion; Jᵥ averaged -0.33±0.03 nl·mm⁻¹·min⁻¹ (Fig. 4, bar b).

In paired experiments, the change in Jᵥ elicited by DBcAMP (ΔJᵥ, condition b vs a) was -0.69±0.02 nl·mm⁻¹·min⁻¹. After washout of DBcAMP, fluid movement in the crypt returned to a rate of absorption that was indistinguishable from that of the initial control period (Fig. 4, bar c).

The effects of CO₂/HCO₃⁻ and DBcAMP on Jᵥ. Fig. 5 presents the effects of 1 mM DBcAMP on fluid movement both when CO₂/HCO₃⁻ was absent and when CO₂/HCO₃⁻ was present in the bath and lumen. Regardless of the CO₂/HCO₃⁻ status, DBcAMP converted net fluid absorption to net fluid secretion. It is interesting to note that the addition of CO₂/HCO₃⁻ to bath and lumen caused a 48% increase in resting rate of fluid absorption (Fig. 5, c vs a) and a 50% increase in the change in net fluid movement induced by DBcAMP (i.e., Fig. 5, conditions b vs a compared with conditions d vs c). Thus ΔJᵥ increased from -0.46±0.04 nl·mm⁻¹·min⁻¹ in the absence of CO₂/HCO₃⁻ to -0.69±0.02 nl·mm⁻¹·min⁻¹ in the presence of CO₂/HCO₃⁻ (P < 0.001). It is possible that CO₂/HCO₃⁻ stimulates absorption and secretion either directly, by promoting HCO₃⁻ transport, or indirectly, by stimulating other transport systems (27).

**Effects of other agonists on Jᵥ.** Since DBcAMP is an intracellular second messenger, experiments were also performed with VIP and ACh, both of which stimulate active Cl⁻ secretion in isolated colonic epithelia (25, 28). Fig. 6 presents the results of the effect of VIP and ACh on Jᵥ, and for comparison includes all of the control (Fig. 6, bar a) and DBcAMP (Fig. 6, bar b) data obtained in the presence CO₂/HCO₃⁻. VIP acts by stimulating adenyl cyclase via receptors on the basolateral membrane (25, 29), while ACh is a neurotransmitter that stimulates active Cl⁻ secretion in both small and large intestine by increasing intracellular [Ca²⁺] which activates basolateral K⁺ channels, hyperpolarizing the cell and promoting Cl⁻ efflux across the
apical membrane (28, 30). Adding 60 nM VIP to the bath converted net fluid absorption to net secretion (Fig. 6, bar c, mean $J_s = -0.35 \pm 0.01 \text{nl} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$). Similarly, addition of 100 $\mu$M ACh to the bath in the presence of CO$_2$/HCO$_3^-$ also induced net fluid secretion (Fig. 6, bar d, mean $J_s = -0.33 \pm 0.03 \text{nl} \cdot \text{mm}^{-1} \cdot \text{min}^{-1}$) at a rate indistinguishable from that produced by either DBcAMP or VIP.

**Discussion**

The spatial-distribution model. The present studies provide evidence that the crypt epithelium of the distal colon has the capacity for both fluid absorption and secretion. Our observations appear to conflict with the established model that secretory processes represent a crypt cell function, whereas absorptive processes are solely a surface epithelial cell function. This established model that has been widely accepted as dogma for 25 yr evolved primarily from circumstantial evidence, without direct measurement of fluid movement in the colonic crypt. The observations that provided the initial basis for this model of specialized function were studies of cholerate enterotoxin in the canine and rabbit small intestine (31, 32). Cycloheximide, a potent inhibitor of protein synthesis, partially inhibited cholerate toxin–induced fluid secretion and decreased mitotic figures in crypts, but did not alter glucose-stimulated fluid absorption (31). Because protein synthesis was thought to occur primarily in crypt cells, these studies were interpreted to mean that fluid secretion, but not fluid absorption, occurs in crypt cells, whereas fluid absorption, and presumably not fluid secretion, occurs in villous cells. In contrast, exposure of jejunal mucosa to hyperosmolar solutions resulted in histologic damage in villous cells and reduced glucose-absorption but did not affect either crypt cell morphology or cholerate toxin–induced fluid secretion (32).

The spatial-distribution model has been supported by other observations. For example, electrophysiology studies on the rabbit distal colon (3) found that luminal application of 0.1 mM amiloride, an inhibitor of apical Na$^+$ channels, increased apical membrane resistance of surface epithelial cells but had no effect in crypt cells. In contrast, PGE$_2$ increased apical membrane conductance in crypt but not surface epithelial cells. Further, PGE$_2$ elicited formation of microscopic fluid droplets in the areas overlying crypt openings but not in areas between crypt openings (3).

However, more recent studies suggest that the spatial-distribution model may be an oversimplification: Villous epithelial cells in the rabbit jejunum exhibit a Cl$^-$–dependent depolarization of apical membrane potentials when exposed to PGE$_2$ (5), and surface epithelial cells in the rat distal colon exhibit a Cl$^-$ conductance when exposed to DBcAMP (6). Although neither study directly demonstrated Cl$^-$ secretion, the work is consistent with the notion that surface/villous cells are capable of secretion. In addition, theoretical considerations led Naftalin et al. to propose that colonic crypt cells absorb a hypertonic fluid (7, 8, 33). As previous studies have not directly assessed the absorptive function of crypt epithelial cells, a method to perfuse colonic crypts was required. Previous experience with both renal tubular and gastric gland microperfusion (17, 20) permitted the development of the present methods for the isolation and microperfusion of hand-dissected colonic crypts. With these methods the resting rate of net fluid movement in isolated crypts could be determined directly.

**Synthesis of the present data with the classical model.** The present observations are not necessarily inconsistent with the classical spatial-distribution model. Our data on isolated colonic crypts, together with results obtained on the T$_{44}$ cell line, which is considered to represent colonic-crypt epithelial cells, suggest a modification of the classical model. In T$_{44}$ cells, active Cl$^-$ transport is absent in the basal state, but active Cl$^-$ secretion is induced by multiple agonists. Interestingly, active Cl$^-$ secretion by T$_{44}$ cells stimulated with bradykinin or serotonin is significantly enhanced when the T$_{44}$ cells are cocultured with an intestinal fibroblast cell line (34). Bradykinin and serotonin induce the release of eicosanoids from these and other nonintestinal fibroblasts (34). These observations suggest that ion secretion in T$_{44}$ cells is not an intrinsic property of the crypt cell in its basal state but rather is induced by paracrine mediators, such as those released from adjacent myofibroblasts and/or other lamina propria cells. The present observation (Fig. 2) that an mAb to myofibroblasts reacts with the native rat distal colon, but not with isolated crypts, is consistent with this model.

An extension of this hypothesis is that Na$^+$–dependent fluid absorption is a constitutive property of isolated colonic crypt epithelial cells. The basolateral surfaces of crypt epithelial cells are in close proximity to several lamina propria cells, including myofibroblasts, immune cells, and enteric neurons. Thus, in the absence of these lamina propria cells and their neurohumoral products, net fluid absorption was present. When agonists that induce active Cl$^-$ secretion were added to the bath solution, crypts were stimulated to secrete. Thus, when situated in their native milieu, crypts are stimulated by the release of one or more neurohumoral agonists from stimulated myofibroblasts and/or other lamina propria cells resulting in the induction of net fluid secretion (35).

The nature of the resting Na$^+$–dependent absorption in these experiments is unknown. The absence of active Na$^+$ absorption in the basal state in T$_{44}$ cells may be, in part, related to their neoplastic origin. Both in vivo perfusion studies and determination of Na$^+$ transport across isolated mucosa of the rat distal colon have established that Na$^+$ absorption is electroneutral and is consistent with Na–H exchange (NHE) (2). Apical membrane vesicles prepared primarily from surface epithelial
cells of the rat distal colon have confirmed the presence of Na–H exchange (36–38). However, Rajendran et al. have recently identified a Na–H exchange with novel properties in apical membrane vesicles prepared from crypt epithelial cells of rat distal colon (38). In these studies, both H + -gradient stimulation of 22Na uptake in crypt apical membrane vesicles and the recovery of intracellular pH from an acid load in epithelial cells require the presence of Cl − (38); thus, the Na–H exchange in the crypt apical membrane is Cl − dependent, unlike all other Na–H exchanges, including that present in surface cell apical membranes. These observations are also consistent with recent in situ hybridization studies that established that the NHE-3 isoform, which has been linked to transepithelial Na + absorption in both rat small and large intestine and rabbit ileum (39, 40), is present only in surface epithelial cells, not in crypt epithelial cells of the rat distal colon (40, 41). Neither the Na–H exchange isoform present in the apical membrane of crypt cells nor the cellular mechanism of Na + absorption in the perfused crypt has been established, but NHE-2 and NHE-4, which have been identified on Northern blot analyses (42, 43), and Cl −-dependent Na–H exchange (38) are likely possibilities.

The observation in experiments on unstirred crypts that the removal of Na + led to net fluid secretion was unexpected (Fig. 3). Because choline was the Na + substitute in these experiments, the observed secretion may have represented the combined effects of both the absence of Na + -dependent absorption and secretion of the organic cation choline (44, 45).

In conclusion, this study presents a newly developed method for the study of isolated colonic crypt epithelial cells with an intact polar orientation. Contrary to the existing concept of the spatial distribution of secretory and absorptive processes to crypt and surface cells respectively, net fluid absorption was identified in the crypt under basal conditions. However, agonists (i.e., VIP and ACh) and intracellular messengers (i.e., DBcAMP) that are known to induce active Cl − secretion in several epithelia reversibly produced net fluid secretion. These results indicate that absorptive processes are constitutively expressed in crypt epithelial cells while secretory ones are regulated by one or more neurohumoral agonists released from lamina propria cells, including myoïbbristles. This methodology will permit studies that will establish the functional properties of colonic crypt epithelial cells.

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