Non-ionic diffusion and carrier-mediated transport drive extracellullar pH regulation of mouse colonic crypts

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1. Extracellular pH (pH_o) regulation within mouse colonic crypt lumens is stimulated by transepithelial gradients of short-chain fatty acids (SCFAs). Current work assesses underlying mechanisms contributing to pH_o regulation.

2. Crypt luminal alkalinization was saturable by apical SCFA (substrate concentration activating half-maximal transport (K_T) of isobutyrate = 45 mM). However, saturation was consistent with either carrier-mediated SCFA flux or non-ionic diffusion, because the non-ionized form was titrated by luminal alkalinization. Direct acidification of apical perfusates increased the magnitude of SCFA-induced luminal alkalinization, roughly in the same proportion to the increased concentration of non-ionized SCFA in the crypt lumen.

3. Transepithelial gradients of an alternative weak acid (CO_2) produce pH_o changes similar to SCFA. In contrast, a weak base (NH_3) changes pH_o with reverse dependence on the orientation of the transepithelial gradient compared with SCFA. Results implicate non-ionic diffusion in pH_o regulation, and suggest that pH_o changes may underly SCFA-stimulated bicarbonate secretion and ammonium absorption.

4. SCFA metabolism plays a minor role in extracellular pH regulation. An avidly metabolized SCFA (*N*-butyrate) augments crypt luminal alkalinization only slightly (0·08 pH units) *versus* a poorly metabolized SCFA (isobutyrate).

5. Apical addition of 1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) partially inhibits luminal alkalinization caused by apical SCFA. DIDS has no effect on luminal alkalinization caused by transepithelial CO_2 gradients. Probenecid (1 mM), α-cyano-4-hydroxycinnamic acid (4 mM) or basolateral DIDS (1 mM) do not affect pH_o regulation. Results suggest that DIDS-sensitive, SCFA-dependent transport in the colonocyte apical membrane contributes to pH_o regulation.

Short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate are produced by bacterial fermentation in the colon (Cummings, Pomare, Branch, Naylor & Macfarlane, 1987; Macfarlane & Cummings, 1991; Macfarlane, Gibson & Cummings, 1992). SCFAs are the major anions in the colonic lumen and are important nutrients for colonocytes (Roediger, 1982; Bergman, 1990; Macfarlane & Cummings, 1991). SCFA metabolism contributes about 7% of total energy maintenance in animals with a simple large intestine (including human), and 80% of energy requirements in ruminants (Engelhardt & Rechkemmer, 1984; Bergman, 1990). SCFAs also stimulate Na^+, Cl^-, NH_4^+, Ca^{2+} and water absorption and bicarbonate secretion, and are thereby important in regulating colonic salt and water conservation (Umesaki, Yajima, Yokokura & Mutai, 1979; Macfarlane & Cummings, 1991; Lutz & Scharrer, 1991; Bödeker, Shen, Kemkowski & Höller, 1992; Dohgen, Hayahshi, Yajima & Suzuki, 1994).

In vivo, a large transepithelial SCFA gradient exists; 100–150 mM total SCFA is in the colonic lumen *versus* less than 0·5 mM in the portal vein (Cummings *et al.* 1987; Bergman, 1990). Evidence suggests that the physiological transepithelial SCFA gradient is important to SCFA action. Over 90% of luminal SCFA is absorbed by the colon, and net SCFA absorption is dependent on the physiological SCFA gradient in some, but not all, colonic segments (Engelhardt & Rechkemmer, 1984; Engelhardt, Burmester, Hansen, Becker & Rechkemmer, 1993). In Ussing chamber experiments, symmetrical exposure to SCFAs often results in net SCFA secretion, not SCFA absorption (Sellin & DeSoignie, 1990; Engelhardt *et al.* 1993; Engelhardt, Gros, Burmester, Hansen, Becker & Rechkemmer, 1994).

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Both non-ionic diffusion and carrier-mediated transport have been proposed to mediate transepithelial SCFA transport in the colon (Argenzio, Southworth, Lowe & Stevens, 1977; Engelhardt & Rechkemmer, 1984; Binder & Metha, 1989; Sellin & DeSoignie, 1990; Sellin, DeSoignie & Burlingame, 1993; Engelhardt *et al.* 1993; Reynolds, Rajendran & Binder, 1993; Rajendran & Binder, 1994). Although transepithelial SCFA transport varies as a function of carbon chain length (i.e. lipophilicity) the relationship is not simply explained by non-ionic diffusion (Salee & Dietschy, 1973; Naupert & Rommel, 1975). Two explanations for the complex behaviour have been suggested. One explanation is that the pH at the surface of the epithelial cells is not the same as in the bulk extracellular fluid, so that rates of non-ionic diffusion are difficult to predict. This has been supported by mathematical modelling of SCFA fluxes (Gutknecht & Tosteson, 1973; Jackson, Williamson, Dombrowski & Garner, 1978; Lucas, 1984) and measurements of pH_o at both the surface epithelium and in colonic crypts (Rechkemmer, Wahl, Kuschinsky & Engelhardt, 1986; Holtug, McEwan & Skadhauge, 1992; Laverty, Holtug, Elbrond, Ridderstråle & Skadhauge, 1994; Chu & Montrose, 1995*a*). Alternatively, carrier-mediated SCFA anion transport has been reported in colonic membranes (including $SCFA^-$–HCO_3^- and $SCFA^-$–Cl^- exchangers) (Reynolds *et al.* 1993; Engelhardt *et al.* 1994; Rajendran & Binder, 1994) or detected immunologically in colon (monocarboxylate–H^+ cotransport) (Garcia, Goldstein, Pathak, Anderson & Brown, 1994). The contribution of these electroneutral SCFA transporters to vectorial SCFA transport remains unknown. As a result, no consensus has been reached about physiologically relevant SCFA transport mechanisms.

SCFA gradients have been shown to generate polarized changes in extracellular pH (pH_o) in microdomains surrounding the colonic crypt epithelium (Chu & Montrose, 1995*a*). Results show that pH_o regulation in crypt lumen is primarily caused by SCFA transport (Chu & Montrose, 1995*a*), but the importance of different SCFA transport mechanisms to this physiological response has not been tested. In this study, we investigate further the effects of physiological SCFA transepithelial gradients on pH_o to help determine which SCFA transport mechanisms may be important.

METHODS

Tissue preparation

CD-1 mice (*Mus musculus*; Charles River, Wilmington, MA, USA) were anaesthetized and killed with halothane vapour, the distal colon excised, flushed with saline, and stripped of muscle layers. Mucosal sheets were kept at 4 °C in Dulbecco's minimum Eagle's medium (Gibco) until use. For experiments, mucosa was mounted in a chamber (Chu & Montrose, 1995*a*) allowing separate superfusion of the apical (mucosal) and basolateral (serosal) surfaces of the tissue while mounted on an inverted microscope (Zeiss Axiovert) coupled to a confocal laser scanning unit (BioRad MRC-600). The serosal side of the tissue was closest to the microscope objective (PlanFluotar × 100, NA 1·2, water immersion; Leitz). The chamber was continuously perfused with solutions at room temperature at a rate of 0·5–1 ml min^{-1} throughout the experiment, using gravity-driven flow.

Perfusate solutions

Perfusate solutions were based on a standard 'Cl^- medium' (containing (mM): 130 NaCl, 5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 20 Hepes, 25 mannose). In SCFA media the NaCl was replaced with equimolar (16–130 mM) sodium isobutyrate or sodium *N*-butyrate. When medium buffering capacity was lowered, 15 mM Hepes was replaced with sodium gluconate.

For experiments with bicarbonate, 20 mM NaCl was replaced with 20 mM $NaHCO_3$ and medium equilibrated with 5% CO_2–95% air to a pH of 7·35–7·40. In these experiments, the bicarbonate-free control medium had 20 mM NaCl replaced with sodium gluconate. Bicarbonate-containing SCFA medium was prepared with 20 mM $NaHCO_3$ and 110 mM sodium isobutyrate. In these experiments, CO_2-impermeable saran tubing (Dow) was used for all perfusion lines.

All media were isosmotic with the original Cl^- medium (range 300–309 mosmol kg^{-1}, Wescor 5500 osmometer), and all solutions were titrated to pH 7·40. When inhibitors were added, the medium pH was retitrated to pH 7·40, if needed.

Measurement of extracellular pH with confocal microscopy

Methods were described previously (Chu & Montrose, 1995*a*). Briefly, muscle-stripped mouse distal colonic epithelium was mounted in a chamber and rapidly superfused with media containing 0·1 mM carboxy SNARF-1 (a pH-sensitive fluorescent dye) throughout the experiment. SNARF-1 was excited by 488 nm laser excitation, and two fluorescence images were collected simultaneously at 640 ± 17 and 580 ± 15 nm. The light path was adjusted daily to optimize a flat-field of collected fluorescence at both wavelengths. Photobleaching of extracellular SNARF-1 was not observed. To equalize *z*-axis resolution between channels, confocal pin holes in front of each detector were set to equal values. All *xy*-images were collected under conditions of constant detector gain and dark current, from a constant plane of focus in each experiment 10–20 μm from the crypt base. Ratio (640/580 nm) images were used to measure pH_o of the crypt lumen (luminal pH), and a manually selected 3–5 μm band in lamina propria directly encircling the crypt basal surface (subepithelial pH), as described previously (Chu & Montrose, 1995*a*).

Statistics

Results are means ± S.E.M. throughout.

RESULTS

Concentration dependency of extracellular pH response to isobutyrate

Experiments examined the response to different SCFA concentrations added in the apical superfusate, to replicate the physiological orientation of SCFA gradients across the colonic epithelium. Isobutyrate was used as a test SCFA because it is only minimally metabolized, and therefore reduces confounding results of SCFA metabolism (Weigand, Young & McGilliard, 1975). Confocal ratio images of extracellular SNARF-1 were used to quantify pH in the crypt lumen and lamina propria surrounding crypts, as described previously (Chu & Montrose, 1995*a*). Figure 1

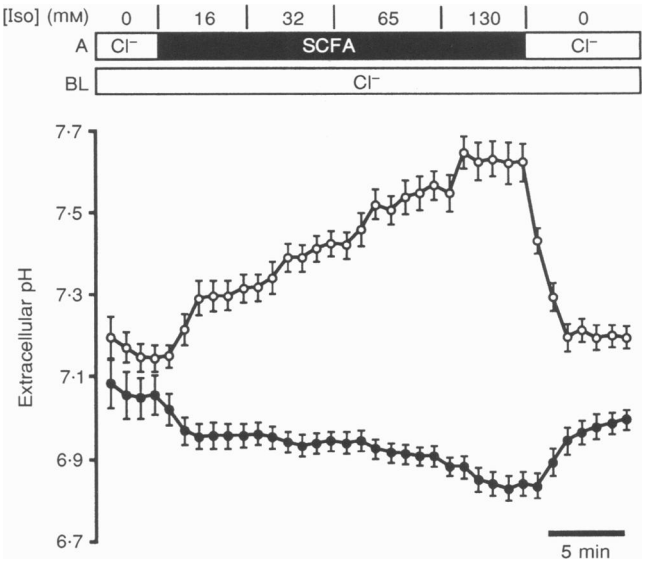

Figure 1. Effect of luminal SCFA concentration on extracellular pH of mouse colonic crypts
Luminal (○) and subepithelial (●) pH values are quantified from ratio (640/580 nm) images. Measurements are expressed as means ± S.E.M. ($n = 11$ crypts in 5 experiments). Results show that sequential addition of 0, 16, 32, 65 and 130 mM sodium isobutyrate (Iso) in apical superfusates caused luminal pH increases from 7·15 ± 0·03 ($n = 11$) to 7·32 ± 0·04, 7·42 ± 0·03, 7·55 ± 0·04 and 7·63 ± 0·05, respectively. Subepithelial pH simultaneously decreased from 7·02 ± 0·04 to 6·96 ± 0·03, 6·94 ± 0·02, 6·91 ± 0·02 and 6·83 ± 0·03 in the same experiments.

shows that both crypt luminal alkalinization and subepithelial tissue acidification are dependent on luminal [isobutyrate]. The magnitude of pH changes in the crypt lumen is larger than in lamina propria. This is probably because the crypt lumen is a small space that allows only slow mixing of apical perfusates, whereas basolateral perfusates are rapidly exchanged in the large lamina propria spaces (Chu & Montrose, 1995*a*). For these reasons, any proton added to the crypt lumen will have a larger effect on pH compared with addition of a proton to the lamina propria.

Figure 2 compiles results from multiple experiments, quantifying the net pH change caused by SCFA. As shown in Fig. 2*A*, the net pH change in the crypt lumen saturates with an increasing [isobutyrate], but in the lamina propria a nearly linear relationship *versus* [isobutyrate] is observed. When results are fitted to the Michaelis–Menten equation by non-linear least squares (GraphPad Software, San Diego, CA, USA), the best-fit parameters of luminal pH change are: substrate concentration activating half-maximal transport (K_T) of isobutyrate = 45 mM and $V_{max} = 0{\cdot}66$ pH units ($r^2 = 0{\cdot}999$). In contrast, pH change in the lamina propria yields a poorer fit ($r^2 = 0{\cdot}968$) in which the best-fit parameters are: K_T of isobutyrate = 95 mM and $V_{max} = -0{\cdot}32$ pH units. The different SCFA dependency of luminal and subepithelial pH implies asymmetry in net acid–base transport at the two sites.

It has been shown previously that SCFA transport mediated (and/or activated) the net proton flux which drives pH_o regulation (Chu & Montrose, 1995*a*). The

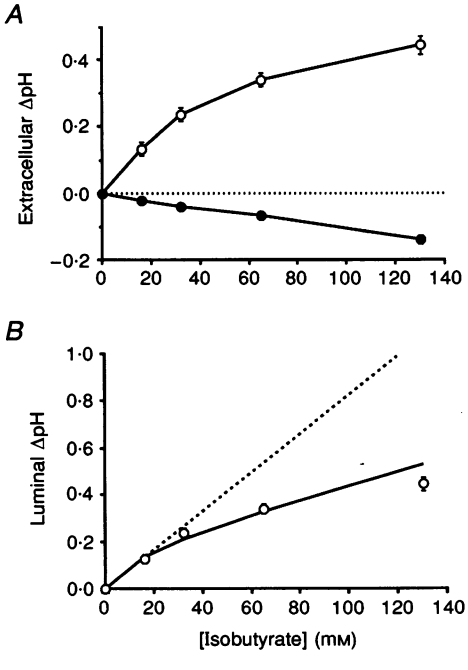

Figure 2. Compiled isobutyrate activation kinetics
Results from experiments in Fig. 1 are compiled and analysed. Plots of extracellular pH change (ΔpH) induced by SCFA *versus* sodium isobutyrate concentration. *A*, SCFA-induced changes in crypt luminal pH (○), or subepithelial pH (●) are plotted. The dotted line is the baseline of no change. *B*, crypt luminal pH change is plotted (○) and compared with two theoretical curves extrapolated from the response to 16 mM isobutyrate. The dotted line is extrapolated from results from non-saturable diffusion. The continuous curve estimates the alteration in non-ionic uptake caused (by titration of the non-ionized form) by the observed luminal pH_o change.

saturable luminal response shown in Fig. 2 could implicate carrier-mediated transport of acid–base equivalents in the response to SCFAs. However, as described below, results can also be fully explained by non-ionic diffusion of SCFA. Since the steady-state luminal pH change caused by SCFAs is due to sustained net proton flux from the lumen (Chu & Montrose, 1995*a*), steady-state pH changes were used to estimate the rate of net proton flux. Figure 2*B* compares the observed luminal pH change (open circles) with two theoretical curves. Both curves assume that (1) all net proton flux is due to non-ionic SCFA uptake from the lumen, and (2) curves can be extrapolated from the observed response to 16 mM SCFA. The dotted straight line is the predicted response when (non-saturable) non-ionic uptake is proportional to the total SCFA concentration in the medium. However, at any given [SCFA], the Hendersen–Hasselbach equation shows that the concentration of the non-ionized form in solution is reduced as pH_0 alkalinizes (see Chu & Montrose, 1995*b*). Using observed pH_0 values to estimate the amount of the non-ionized form at each [SCFA], the continuous curve shows the predicted response due to non-ionic SCFA uptake after correction for titration of the non-ionic form. Comparison of the two curves indicates that the observed luminal alkalinization will significantly blunt non-ionic uptake, to produce an apparent saturation quantitatively similar to that observed in experiments. This implies that the observation of 'saturable' proton fluxes cannot be used to distinguish the contribution of non-ionic diffusion *versus* carrier-mediated transport.

As a more stringent test for non-ionic diffusion, experiments were performed that imposed changes in apical pH to directly alter the concentration of non-ionized SCFA. Apical superfusates were titrated to pH 6·5, 7·0 and 7·5. Figure 3*A* presents values of crypt luminal pH in these different conditions in the absence (open circles) or the presence of SCFA (filled circles). In these experiments, the same crypts were exposed to all conditions (i.e. each crypt is its own control for all different conditions). As shown, the crypt lumen was able to partially resist changes in superfusate pH even in the absence of SCFA (1 pH unit change in superfusate led to 0·3 pH unit change in the crypt lumen). This could be due to endogenous luminal buffering capacity, changes in cellular acid/base extrusion into the crypt lumen, and/or paracellular flux of base equivalents from the relatively alkaline basolateral environment. Results were not explained by alterations in perfusate pH, because the pH of chamber effluents was checked to confirm that no medium pH changes had occured during an experiment. In the presence of SCFA (filled circles in Fig. 3*A*), crypt luminal pH was more stable against changes in superfusate pH (1 pH unit change in superfusate led to 0·07 pH unit change in the crypt lumen) *versus* the absence of SCFA. This suggests that the presence of luminal SCFAs helps the crypt resist pH changes in the colonic lumen.

The results in Fig. 3*A* were used to quantify the magnitude of SCFA-induced pH changes at different pH_0 values. The goal was to ascertain if the magnitude of luminal alkalinization caused by SCFAs is proportional to the concentration of non-ionized form. The filled bars in Fig. 3*B* show the measured alkalinization of luminal pH due to SCFA addition, at each perfusate pH. As shown, the ΔpH is modestly increased at more acidic values of pH_0. This is qualitatively consistent with an increased concentration of non-ionized SCFA at lower pH values. The hatched bars are the predicted change in pH_0 due to non-ionic diffusion, assuming (1) the crypt luminal pH observed in the absence of SCFA can be used to estimate the initial concentration of non-ionized SCFA available for crypt transport, and (2) the magnitude of SCFA-induced ΔpH is proportional to the initial concentration of the non-ionized form. Results are presented relative to the results when perfusate pH = 7·5. That is, we used results at pH 7·5 to predict the changes that should be observed at perfusate pH 7·0 and 6·5. As shown, there is a good match between observed and predicted values, suggesting non-ionic diffusion of SCFAs can explain results.

Effect of transepithelial weak acid or base gradients on extracellular pH

To address the capability of non-ionic diffusion to drive pH_0 regulation more directly, experiments were designed to see if other weak acids or bases could produce similar effects to SCFAs. Experiments first explored if another weak acid

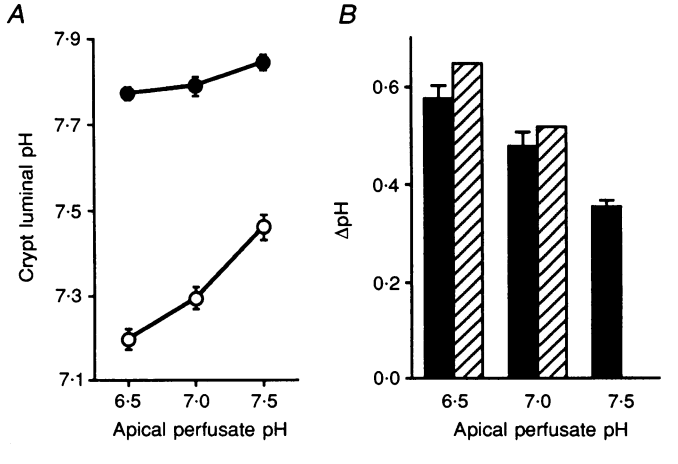

Figure 3. Effect of varying apical perfusate pH on crypt luminal pH

A, crypt luminal pH measured during apical superfusion with medium of pH 6·5, 7·0 or 7·5. Values were recorded after 7–10 min incubation at the indicated pH in chloride media (○) or SCFA media with 130 mM sodium isobutyrate (●); $n = 24$ crypts. *B*, crypt luminal pH change (ΔpH) induced by SCFA at the indicated superfusate pH. Filled bars are measured ΔpH values in the crypt lumen ($n = 24$). Hatched bars are the predicted ΔpH values for pH 6·5 and 7·0 superfusate caused by non-ionic diffusion of SCFA (see text).

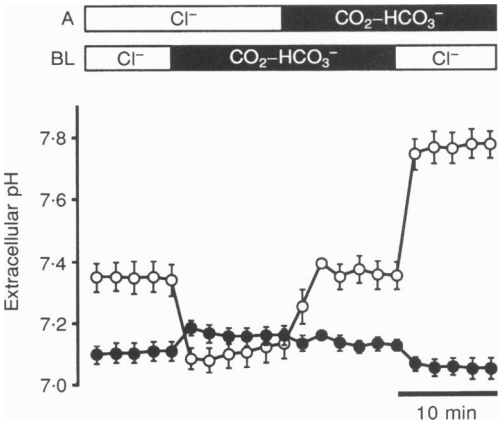

Figure 4. Effect of transepithelial CO_2–HCO_3^- gradients on extracellular pH

Time course of crypt extracellular pH while epithelia exposed to Cl^- medium (Cl^-) or 20 mM bicarbonate medium (CO_2–HCO_3^-) in apical (A) and/or basolateral (BL) superfusates, as indicated. Results compiled separately for measurements of luminal (○) and subepithelial pH (●). $n = 7$ crypts from 3 experiments.

(CO_2) could replicate observations made with SCFAs. Our previous study had shown that transepithelial SCFA gradients, and not the simple presence of SCFAs, was required to generate polarized changes in extracellular pH (Chu & Montrose, 1995*a*). Therefore we asked if transepithelial CO_2–HCO_3^- gradients affected pH_o of colonic crypts. Figure 4 compiles time course experiments in which epithelia were sequentially exposed to medium containing 20 mM bicarbonate (5% CO_2, pH 7·35–7·40) in basolateral and/or apical perfusates. Results show that reversing the orientation of the transepithelial CO_2–HCO_3^- gradient produces opposite changes in luminal and subepithelial pH while bilateral superfusion with identical CO_2–HCO_3^--containing medium minimizes changes in pH_o. These results suggest that transepithelial gradients of another weak acid (CO_2–HCO_3^-) causes polarized pH_o changes qualitatively similar to SCFAs.

Results were compared with the effects of the weak base ammonium. Non-ionic diffusion of a weak base is predicted to cause polarized pH_o change which has the opposite dependence on the orientation of a transepithelial gradient compared with weak acids. This is because the ionic form (NH_4^+) is acidic, and the non-ionic form (NH_3) is basic. To test this prediction, colonic epithelium was sequentially exposed to 25 mM NH_4Cl (pH 7·4) in basolateral and/or apical superfusates. As shown in Fig. 5, reversing the orientation of the transepithelial NH_4Cl gradient produces opposite changes in luminal pH, and bilateral exposure to identical [NH_4Cl] minimized changes in luminal pH. The qualitative response to transepithelial NH_3–NH_4^+ gradients was opposite to the effects of SCFAs and CO_2–HCO_3^-. This is consistent with our prediction about weak base non-ionic diffusion at the luminal membrane. However, we did not anticipate that subepithelial pH would be unchanged when the epithelium was exposed to basolateral NH_4Cl. This may be caused by active NH_4^+ transport into colonocytes via basolateral Na^+,K^+-ATPase (Towle & Hølleland, 1987), which could neutralize the pH_o acidification caused by non-ionic NH_3 uptake. Results from both ammonium and CO_2 experiments suggest that rapid transport of weak acid–base pairs across the epithelium is capable of eliciting pH_o changes.

Effect of SCFA metabolism on extracellular pH regulation

Experiments were designed to test if factors other than transport contributed to pH_o regulation in colonic crypts. To study the effect of SCFA metabolism on pH_o, experiments compared *N*-butyrate with isobutyrate. The two SCFAs have the same pK_a values and are equally transported across intestinal membranes (Weigand *et al.* 1975; Jackson *et al.* 1978; Gabel, Vogler & Martens, 1991; Chu & Montrose, 1995*b*), but *N*-butyrate is a major

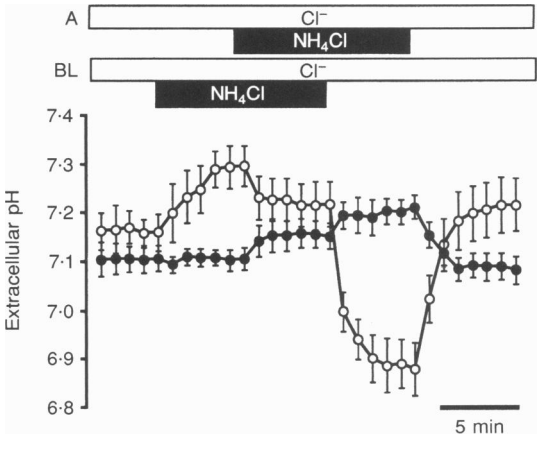

Figure 5. Effect of transepithelial NH_3–NH_4^+ gradients on extracellular pH

Time course of crypt extracellular pH while epithelia are exposed to Cl^- medium (Cl^-) or 25 mM NH_4Cl added to Cl^- medium (NH_4Cl) in apical (A) and/or basolateral (BL) superfusates, as indicated. Results compiled separately for measurements of luminal (○) and subepithelial pH (●). $n = 10$ crypts from 3 experiments.

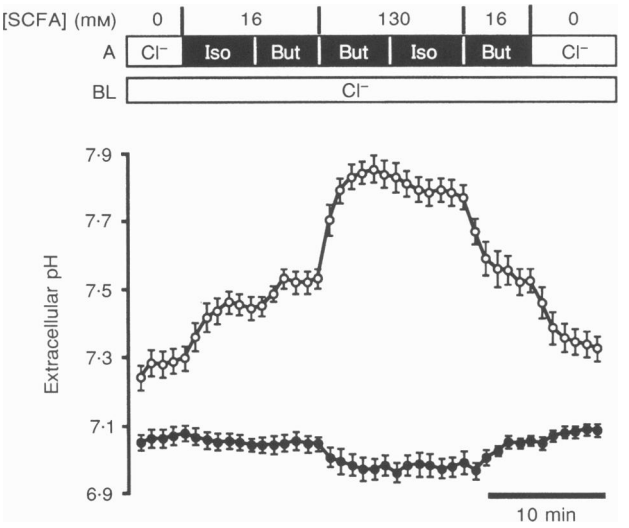

Figure 6. Effect of cellular metabolism of SCFAs on extracellular pH

Time course of crypt extracellular pH while epithelia exposed to isobutyrate (Iso) or *N*-butyrate (But) of known concentration (16 and 130 mM) in apical superfusate. Results compiled separately for measurements of luminal (○) and subepithelial pH (●). $n = 7$ crypts from 3 experiments.

metabolic fuel of colonocytes while isobutyrate is negligibly metabolized (Weigand *et al.* 1975; Roediger, 1982). Our previous observations had hinted that quantitative differences in the physiological response to these two SCFAs may exist (Chu & Montrose, 1995*a*). Therefore new experiments directly compared effects of *N*-butyrate and isobutyrate at both low and high concentration (16 and 130 mM) in the same tissue. Luminal addition of 16 mM isobutyrate alkalinized the crypt lumen (steady-state $pH_o = 7{\cdot}46 \pm 0{\cdot}03$, mean ± S.E.M., $n = 7$ crypts) and tended to cause acidification in lamina propria (Fig. 6). Switching to 16 mM *N*-butyrate produced a small further alkalinization ($pH_o = 7{\cdot}53 \pm 0{\cdot}03$) which was significantly greater than results from equimolar isobutyrate ($P = 0{\cdot}016$ in two-tailed Student's paired t test). Luminal superfusates were then switched to 130 mM SCFA. At this concentration *N*-butyrate also produced greater luminal alkalinization than equimolar isobutyrate ($pH_o = 7{\cdot}84 \pm 0{\cdot}04$ *versus* $7{\cdot}79 \pm 0{\cdot}04$, $P = 0{\cdot}004$). Subepithelial pH did not change detectably during the switch between *N*-butyrate and

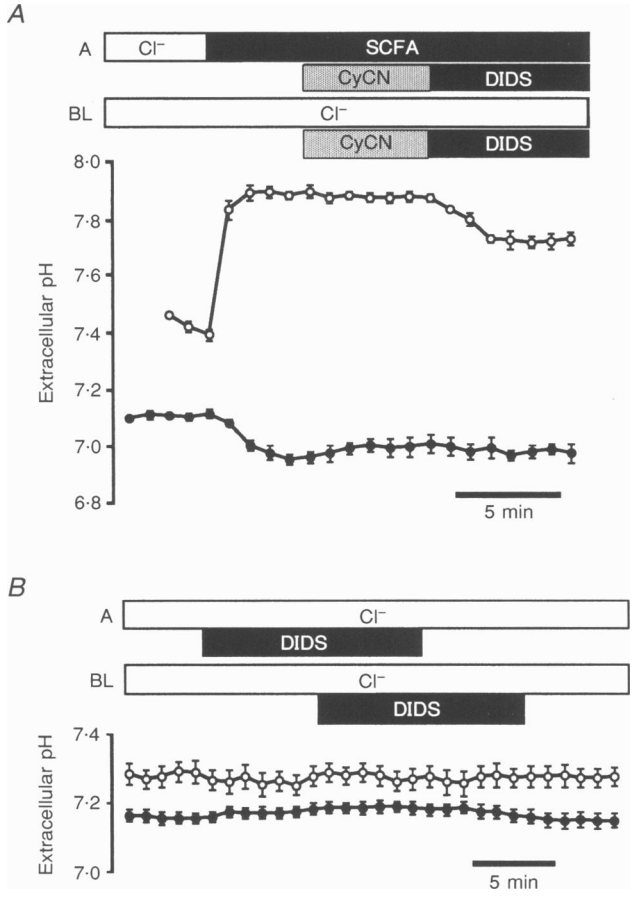

Figure 7. DIDS, but not CyCN, partially inhibits extracellular pH regulation

A, time course of crypt extracellular pH while epithelia are exposed to 130 mM sodium isobutyrate in apical perfusate. At indicated times, either 4 mM CyCN or 1 mM DIDS was present in apical and basolateral perfusates. Results are compiled separately for measurements of pH in luminal (○) and subepithelial (●) compartments. $n = 3$ crypts from a representative experiment. Qualitatively similar results were observed in 2 experiments. *B*, effect of DIDS during bilateral superfusion with Cl^- medium. Extracellular pH recorded after epithelia exposed to 1 mM DIDS in apical (A) and/or basolateral (BL) superfusates, as indicated. $n = 8$ crypts from 2 experiments.

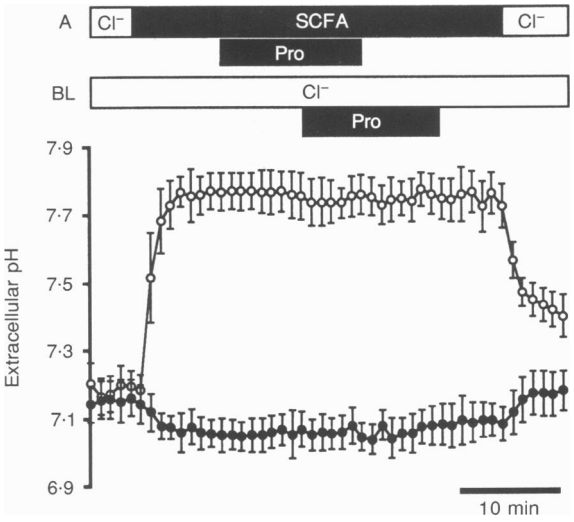

Figure 8. Probenecid does not affect extracellular pH regulation

Time course of extracellular pH after exposure to apical 130 mM sodium isobutyrate (SCFA) and subsequent exposure to 1 mM probenecid (Pro) in apical (A) and/or basolateral (BL) superfusates, as indicated. Results compiled separately for measurements of luminal (○) and subepithelial pH (●). $n = 4$ crypts from 2 experiments.

isobutyrate, although subepithelial acidification was evident at 130 mM SCFA. The magnitude of the luminal pH change caused by switching between isobutyrate and *N*-butyrate was similar ($P = 0{\cdot}241$) at both low ($\Delta pH = 0{\cdot}08 \pm 0{\cdot}02$) and high ($\Delta pH = 0{\cdot}05 \pm 0{\cdot}01$) SCFA concentrations. The similar ΔpH was observed despite a 3-fold difference in the overall luminal pH excursion (see Fig. 1), indicating that the differential effect of *N*-butyrate (potentially due to metabolism) was maximal by 16 mM.

Effect of transport inhibitors on extracellular pH microdomain

We have previously shown that sustained net vectorial proton flux is activated in the presence of apical SCFAs. Therefore, experiments were carried out to test the effects of inhibitors on the steady-state pH_o generated by apical SCFA (isobutyrate). α-Cyano-4-hydroxycinnamic acid (CyCN) is a relatively specific inhibitor of monocarboxylate–H^+ co-transport (Edlund & Halestrap, 1988; Poole, Halestrap, Price & Levi, 1989; Garcia *et al.* 1994), but 4 mM CyCN added to both apical and basolateral superfusion did not affect the response to isobutyrate (Fig. 7*A*). In the same tissues, 1 mM 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS, a broad spectrum anion transport inhibitor) resulted in a significant decline of luminal pH from $7{\cdot}88 \pm 0{\cdot}03$ to $7{\cdot}71 \pm 0{\cdot}04$ ($n = 3$, $P = 0{\cdot}006$). Control experiments showed that pH_o was not affected by 1 mM DIDS in the absence of SCFA (Fig. 7*B*). Results show that DIDS affected the proton net transport activated by the SCFA. Experiments were also carried out to test if probenecid (an inhibitor of renal organic anion transport) (Guggino & Guggino, 1989) affected pH_o. Results indicate that neither apical nor basolateral probenecid (1 mM) changed extracellular pH (Fig. 8).

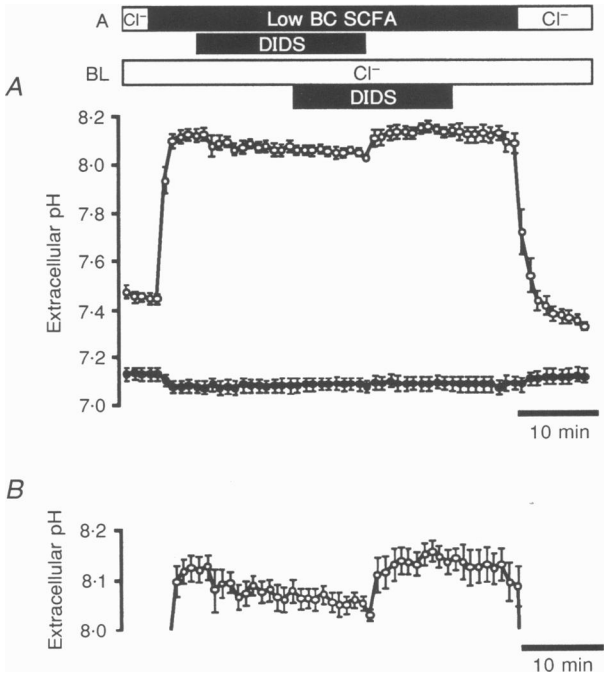

Figure 9. Polarity of DIDS action on extracellular pH regulation

Time course of extracellular pH after exposure to apical 100 mM sodium isobutyrate with low buffering capacity (Low BC SCFA) and subsequent exposure to 1 mM DIDS (DIDS) in apical (A) and/or basolateral (BL) superfusates, as indicated. DIDS in apical superfusate partially inhibits SCFA-induced luminal alkalinization. Basolateral DIDS shows no effect on the extracellular pH regulation. Results compiled separately for measurements of luminal (○) and subepithelial pH (●). $n = 6$ crypts from 3 experiments.

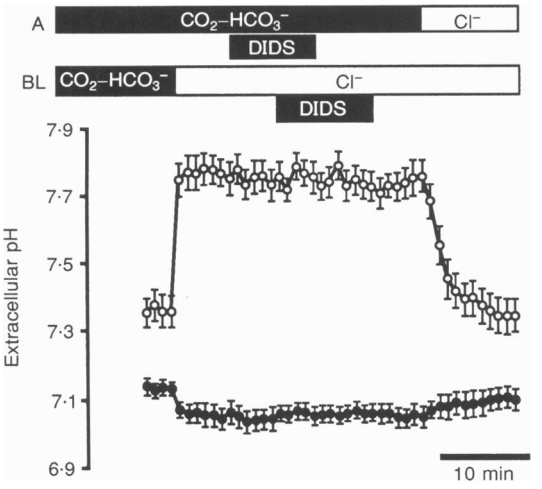

Figure 10. DIDS does not affect pH_o in the presence of a CO_2–HCO_3^- transepithelial gradient

Time course of pH_o after exposure to 20 mM bicarbonate buffer (equilibrated with 5% CO_2–95% air) in apical (A) superfusate and subsequent exposure to 1 mM DIDS in apical and/or basolateral (BL) superfusates. The polarized extracellular pH change caused by transepithelial CO_2–HCO_3^- gradient is not affected by 1 mM DIDS. Results compiled separately for measurements of luminal (○) and subepithelial pH (●). $n = 7$ crypts from 3 experiments.

Further experiments focused on characterizing the effect of DIDS. Apical medium with low buffering capacity was used to increase the magnitude of pH_o excursions caused by luminal SCFA (Chu & Montrose, 1995*a*); to potentially optimize the ability to detect DIDS-sensitive events by lowering non-ionic SCFA uptake (by alkaline titration of the non-ioinized form). Figure 9*A* shows both luminal and subepithelial pH_o on the same graph, whereas Fig. 9*B* presents the same luminal pH results on a magnified pH_o scale (but the same time scale). Results in Fig. 9 show that DIDS reversibly inhibited luminal alkalinization when added to the apical superfusate, but basolateral DIDS had no effect. The steady-state pH_o (8·12 ± 0·03; $n = 6$) was significantly decreased by addition of DIDS (8·07 ± 0·02; $P = 0·001$, paired t test). Basolateral acidification caused by luminal isobutyrate was not affected by either apical or basolateral DIDS. Results suggest that the DIDS-sensitive acid–base transporter is at the apical membrane of the epithelium, but that its activity is a minor component of pH_o regulation even when non-ionic diffusion is minimized.

DIDS effect on extracellular pH is SCFA dependent

Experiments used transepithelial CO_2–HCO_3^- gradients to generate polarized changes of pH_o in the absence of SCFAs. As shown in Fig. 10, DIDS had no significant effect on pH_o although values of pH_o closely paralleled those produced by SCFAs. This suggests that the DIDS-sensitive transporter is not simply activated by changes in pH_o, and that a DIDS-sensitive SCFA transporter may be involved in pH_o regulation.

Figure 11 shows the response to SCFAs in the presence of CO_2–HCO_3^-. In this experiment, the only imposed transepithelial gradient is of SCFA; CO_2–HCO_3^- is present in all superfusates at both membrane surfaces. As shown, steady-state pH_o before DIDS addition was 7·44 ± 0·02 ($n = 6$), and significantly decreased to 7·37 ± 0·02 in the presence of apical DIDS ($P = 0·001$). After removal of apical DIDS (but in the continued presence of basolateral DIDS), pH_o returned to 7·43 ± 0·01 ($P = 0·001$ *versus* presence of apical DIDS, $P = 0·364$ *versus* pH_o before DIDS

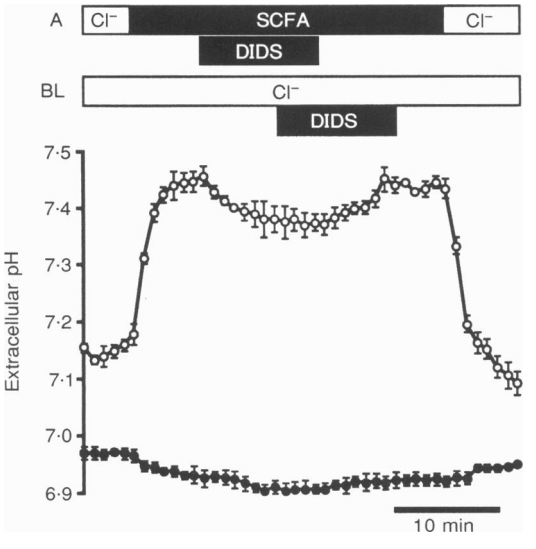

Figure 11. Polarity of DIDS action on extracellular pH regulation of SCFA in bicarbonate buffer

All solutions contained 20 mM bicarbonate and were equilibrated with 5% CO_2–95% air atmosphere. Time course of extracellular pH after exposure to apical 130 mM sodium isobutyrate (SCFA) and subsequent exposure to 1 mM DIDS (DIDS) in apical (A) and/or basolateral (BL) superfusates, as indicated. DIDS in apical superfusate partially inhibits SCFA-induced luminal alkalinization. Results compiled separately for measurements of luminal (○) subepithelial pH (●). $n = 6$ crypts from 3 experiments.

addition). These results suggest that (1) pH_o regulation is observed in physiological buffering conditions, (2) apical DIDS is competent to act as an inhibitor in the presence of CO_2–HCO_3^- (an important control for Fig. 10), and (3) addition of CO_2–HCO_3^- does not enhance pH_o regulation compared with in the absence of CO_2–HCO_3^-. The last point suggests that both the DIDS-sensitive transport and the overall action of SCFAs on pH_o are bicarbonate independent.

DISCUSSION

Previous work demonstrated that vectorial SCFA transport caused polarized extracellular pH regulation (Chu & Montrose, 1995*a*). This conclusion is extended by the present results. As detailed below, evidence suggests non-ionic diffusion of SCFAs is sufficent to explain the major portion of pH_o regulation in the crypt lumen. The saturation of crypt luminal alkalinization at high [SCFA] closely matches theoretical predictions of the magnitude of non-ionic SCFA uptake, after correction for observed changes in luminal pH_o. In addition, imposed pH changes in the apical perfusate affect the magnitude of SCFA-induced luminal alkalinization roughly proportion to the amount of non-ionized SCFA present in the crypt lumen. Finally, results with another weak acid (CO_2) and a weak base (ammonium) also suggest non-ionic diffusion can affect crypt luminal pH_o regulation.

Results in Fig. 3 suggest that SCFAs will help the crypt lumen maintain a relatively constant pH_o despite wide changes in pH of the colonic lumen. Earlier experiments measuring the pH sensitivity of transepithelial SCFA fluxes were probably confounded by this pH_o constancy (Engelhardt & Rechkemmer, 1984; Sellin & DeSoignie, 1990; Sellin *et al.* 1993). It is simple to envisage how non-ionic diffusion could mediate this pH_o regulation in the crypt lumen. Luminal alkalinization could be produced by non-ionic SCFA influx into colonocytes, when extracellular protons are consumed (via re-association of SCFA anions with protons) to fuel continuous non-ionic SCFA uptake (Gutknecht & Tosteson, 1973). Basolateral efflux of SCFA (via non-ionic diffusion or other mechanisms) would permit sustained SCFA uptake at the apical membrane, but the luminal alkalinization would eventually slow uptake by titration of the non-ionized form (as suggested by the kinetic analysis in Fig. 1). Thus results suggest that luminal alkalinization may act as a negative feedback loop controlling SCFA uptake.

Non-ionic diffusion was reported to be a minor component of SCFA fluxes in isolated apical membrane vesicles from rat colonocytes (Mascolo, Rajendran & Binder, 1991). As summarized below, our results identify two events besides non-ionic diffusion which also have an impact on observations, but evidence suggests that these processes make only minor contributions to pH_o regulation. The basis for such differences between mouse and rat colonocytes, and two different experimental preparations, has yet to be resolved. One possibility is that the contribution of non-ionic uptake to vesicular transport may have been minimized by rapid equilibration of the non-ionized form across the vesicle membrane, and/or limited by resultant acidification of intravesicular pH.

In mouse colonocytes, SCFA metabolism and a SCFA-activated DIDS-sensitive transporter in the apical membrane affected pH_o regulation. The DIDS-sensitive transport is comparable either in the absence or presence of bicarbonate, so the DIDS-sensitive transporter is unlikely to mediate SCFA–HCO_3^- exchange. Assuming that transport is not saturated by atmospheric CO_2 (which generates 0·3 mM HCO_3^- in pH 7·4 solution), our results suggest that the DIDS-sensitive process is bicarbonate independent. Lack of inhibition by α-cyano-4-hydroxycinnamic acid suggests that monocarboxylate–proton cotransport is not involved in pH_o regulation, but the identity of the DIDS-sensitive transport process in mice is unknown. Stilbene-sensitive SCFA transport has been previously observed in HT29 cells (Rowe, Blackmon & Montrose, 1993), and stilbene-sensitive $SCFA^-$–HCO_3^- and $SCFA^-$–Cl^- exchange in some intestinal membranes (Harig, Soergel, Barry & Ramaswamy, 1991; Reynolds *et al.* 1993; Rajendran & Binder, 1994). Overall, results support the suggestion that both carrier-mediated SCFA transport and SCFA metabolism are participants in pH_o regulation, albeit to a small extent.

Luminal SCFAs stimulate colonic bicarbonate secretion (Argenzio *et al.* 1977; McNeil, Cummings & James, 1978; Umesaki *et al.* 1979; Ruppin, Bar-meir, Soergel, Wood & Schmitt, 1980; Dohgen *et al.* 1994) and ammonium absorption (Bödeker *et al.* 1992). Our results strongly suggest that a transepithelial pH gradient is one driving force underlying SCFA-stimulated ammonium absorption and bicarbonate secretion. We have shown that SCFAs are competent to drive pH_o regulation in the presence of CO_2–HCO_3^-. We have also demonstrated that either CO_2 or NH_3 gradients can directly move net acid/base equivalents across the epithelium. Therefore, the transepithelial pH gradient established by SCFAs will stimulate vectorial transport of these weak acid/bases, and will also promote net (vectorial) titration of the compounds across the epithelium in the appropriate direction.

In summary, results demonstrate multiple pathways controlling pH_o regulation and SCFA transport in colonic crypts. Both non-ionic diffusion and carrier-mediated (DIDS-sensitive) transport are likely to play a role in these functions. Results establish a role for colonocytes and SCFAs in regulating the luminal environment of the colonic crypts.

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