An Na\textsuperscript{+}-independent Short-Chain Fatty Acid Transporter Contributes to Intracellular pH Regulation in Murine Colonocytes

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ABSTRACT Short-chain fatty acids (SCFAs) are the major anions in the colonic lumen. Experiments studied how intracellular pH (pHi) of isolated colonocytes was affected by exposure to SCFAs normally found in the colon. Isolated crypt fragments were loaded with SNARF-1 (a fluorescent dye with pH-sensitive excitation and emission spectra) and studied in a digital imaging microscope. Intracellular pH was measured in individual colonocytes as the ratio of fluorescence intensity in response to alternating excitation wavelengths (575/505 nm). After exposure to 65 mM acetate, propionate, \( n \)-butyrate, or \( \alpha \)-butyrate in isosmotic Na\textsuperscript{+}-free media (substituted with tetramethylammonium), all colonocytes acidified rapidly and then >90% demonstrated a pHi alkalinization (Na\textsuperscript{+}-independent pH\textsubscript{i} recovery). Upon subsequent removal of the SCFA, pH\textsubscript{i} alkalinized beyond the starting pH\textsubscript{i} (a pH\textsubscript{i} overshoot). Using propionate as a test SCFA, experiments demonstrate that the acidification and pH\textsubscript{i} overshoot are explained by transmembrane influx and efflux of nonionized SCFA, respectively. The basis for the pH\textsubscript{i} overshoot is shown to be accumulation of propionate during pH\textsubscript{i} alkalinization. The Na\textsuperscript{+}-independent pH\textsubscript{i} recovery (a) demonstrates saturable propionate activation kinetics; (b) demonstrates substrate specificity for unmodified aliphatic carbon chains; (c) occurs after exposure to SCFAs of widely different metabolic activity, (d) is electroneutral; and (e) is not inhibited by changes in the K\textsuperscript{+} gradient, Cl\textsuperscript{−} gradient or addition of the anion transport inhibitors DIDS (1 mM), SITS (1 mM), \( \alpha \)-cyano-4-hydroxycinnamate (4 mM), or probenecid (1 mM). Results suggest that most mouse colonocytes have a previously unreported SCFA transporter which mediates Na\textsuperscript{+}-independent pH\textsubscript{i} recovery.

INTRODUCTION

Bacterial fermentation of undigested carbohydrate and protein produces short-chain fatty acids (SCFAs) in the mammalian colon or rumen (Bugaut, 1987; Bergman, 1990; Macfarlane and Cummings, 1991; Macfarlane, Gibson, and Cummings, 1992).
In normal mammals, the colonic lumen contains 100–150 mmol/liter total SCFAs (Wrong, Metcalfe-Gibson, Morrison, Ng, and Howard, 1965; Hoverstad, Midthvedt, and Bohmer, 1985; Hoverstad and Midthvedt, 1986; Cummings, Pomare, Branch, Naylor, and Macfarlane, 1987). The molar ratio among the three major SCFAs (which constitute roughly 90% of the SCFAs in the lumen) varies due to a number of factors but overall is ~60:20:20 for acetate:propionate:butyrate (Cummings et al., 1987; Weaver, Krause, Miller, and Wolin, 1989; Macfarlane and Cummings, 1991). In mammals, 95–99% of SCFAs produced in the colonic lumen are absorbed (Engelhardt and Rechkemmer, 1985; Macfarlane and Cummings, 1991). Because the molar ratios found in the lumen approximate those produced during bacterial fermentation (Holtug, Rasmussen, and Mortensen, 1992), all the major SCFA species must be avidly absorbed.

The route of colonic SCFA absorption is debated, but has been shown to involve primarily transcellular, and not paracellular, fluxes (Engelhardt and Rechkemmer, 1992). Early work suggested the presence of nonionic diffusion as a major mechanism for transmembrane SCFA flux in intestine and colon (Naupert and Rommel, 1975; Schmmit, Soergel, and Wood, 1976; Jackson, Williamson, Dombrowski, and Garner, 1978; Ronnau, Guth, and Engelhardt, 1989). Such fluxes (with the resultant cellular acidification) have been supported as the mechanism by which SCFAs strongly stimulate apical Na+/H+ exchange to promote electroneutral sodium absorption (Petersen, Wood, Schulze, and Heintze, 1981; Holtug, 1989; Binder and Mehta, 1989; Gabel, Vogler, and Martens, 1991; Sellin and DeSoigne, 1990; Rowe, Lesho, and Montrose, 1994). However, SCFAs are weak acids, and the most abundant SCFAs in the colonic lumen all have pKₐ values of 4.8–4.9. This implies that at the prevailing pH values of the colonic lumen (pH 6–7.5; Cummings et al., 1987), at least 90% of all SCFAs exist in the ionized (anionic) form. Despite a relative abundance of the anionic form, it is difficult to predict whether the transmembrane fluxes of any one molecular form will predominate because nonionic diffusion is extremely rapid across many artificial and cellular membranes (Roos and Boron, 1981; Walter and Gutknecht, 1984; Montrose and Kimmich, 1986).

Recent work has suggested that nonionic diffusion of SCFAs may be small compared to other mechanisms of SCFA transport across colonic membranes. In intact tissue and isolated intestinal membranes, the effect of increasing carbon chain length (Engelhardt and Rechkemmer, 1992) or lowering medium pH (Mascolo, Rajendran, and Binder, 1991; Harig, Soergel, Barry, and Ramaswamy, 1991) did not increase SCFA flux as predicted from nonionic diffusion models. However, mathematical modeling of transepithelial SCFA fluxes suggests that it may be difficult at the whole tissue level to distinguish between ionic transport of SCFAs versus nonionic diffusion which is perturbed by local microdomains of pH within, or adjacent to, the epithelium (Jackson et al., 1978).

In addition, extensive work with intact tissue and isolated membrane vesicles has identified several mechanisms mediating flux of SCFA anions. Experiments have suggested the presence of electroneutral SCFA⁺/HCO₃⁻ or SCFA⁺/Cl⁻ exchange in small intestine, gallbladder, and colon (Petersen et al., 1981; Harig, Soergel, Barry, and Ramaswamy, 1991; Mascolo et al., 1991; Gabel et al., 1991; Reynolds, Rajendran, and Binder, 1993). Similarly Na⁺/monocarboxylate and H⁺/monocarboxylate
cotransporters have also been reported in cells derived from intestinal and/or renal epithelia (Siebens and Boron, 1987; Nakhoul and Boron, 1988; Nakhoul, Lopes, Chaillet, and Boron, 1988; Rosenberg, Fadil, and Schuster, 1993; Garcia, Goldstein, Pathak, Andersons, and Brown, 1994). The physiologic roles of these SCFA ion transporters have yet to be established, although it has been suggested that these transporters, and not nonionic diffusion, may function to acidify colonocytes (Rajendran and Binder, 1994). At a whole tissue level, it seems possible that transcellular flux of both nonionized and ionized SCFAs will contribute to the final colonic response to SCFAs in terms of stimulating transepithelial transport of ions and SCFAs themselves.

Because SCFAs are weak acids, transmembrane flux of either the nonionized or ionized form is predicted to play a major role in affecting colonocyte pH (Roos and Boron, 1981). Therefore, colonocytes should have effective machinery to support intracellular pH (pHi) homeostasis and insure normal physiologic and metabolic functions of the cell despite constant exposure to SCFAs. The cellular physiology of the colonocyte pHi response to SCFAs is poorly understood. Activation of Na+/H+ exchange, as suggested from numerous studies (Petersen et al., 1981; Holtug, 1989; Binder and Mehta, 1989; Gabel et al., 1991), will act to regulate pHi. However in addition, all of the SCFA transporters identified are predicted to mediate net acid/base transport either directly or indirectly. Our goal in this manuscript was to evaluate mechanisms by which transport of SCFAs affects colonocyte pH.

In the present study, we report measurements of pHi from isolated murine colonocytes loaded with SNARF-1 (Bassnett, Reinisch, and Beebe, 1990; Buckler and Vaughan-Jones, 1990). Using digital fluorescence imaging and dual excitation ratioing to measure pHi, we observe both Na+-independent and Na+-dependent pHi recovery in response to the cellular acidification caused by physiological concentrations of SCFAs in the medium. Results suggest that uptake of nonionized SCFA causes acidification of colonocytes and that an electroneutral SCFA transporter mediates Na+-independent pHi alkalinization to promote recovery of pHi. The SCFA transporter demonstrates substrate specificity favoring naturally occurring SCFAs, and has characteristics which distinguish the transporter from previously identified SCFA transport mechanisms.

METHODS

Isolation of Colonocytes

CD-1 mice of 19–21 g (Charles River Laboratories, Wilmington, MA) were sacrificed by exposure to Halothane vapor (Halocarbon Laboratories, River Edge, NJ) in a desiccator. A midline incision was made to open the abdomen and the entire colon distal to the cecum was removed from the mouse. Preparation of isolated mouse colonocytes was based on a simplification of the method of Kaunitz (1988). A syringe was used to flush the colonic lumen with 30 ml of ice cold Hank’s buffered saline solution (GIBCO-BRL, Gaithersburg, MD) containing 0.015% dithiothreitol and 1% bovine serum albumin. The colonic mucosa was exposed by a cut along the mesenteric line, kept moist with Hank’s solution, and the mucosa isolated from muscularis and submucosal tissue by gentle scraping with a razor blade. The colonic mucosa was minced by cross-cutting with two razor blades and then incubated in 50 ml of Dulbecco’s modified Eagle medium (DMEM) containing 0.1% collagenase (type IA, Sigma Chemical Co.,
St. Louis, MO) for 60 min at 37°C with gentle agitation. The digested mucosa was filtered through a nylon mesh to separate isolated colonocytes and intact crypts from undigested tissue. The filtered solution was centrifuged at 500 g for 10 min. Supernatant was discarded and the pellet was resuspended with DMEM to 10-14 ml in a 15 ml conical plastic tube. After room temperature sedimentation (at 1 g) for 10 min, supernatant was aspirated down to the last 0.5 ml. The remaining solution containing isolated colonocytes and colonic crypts was kept on ice. Aliquots of this material were used for experiments over 4-5 h with no detectable change in function or viability (data not shown).

**Measurement of Intracellular pH**

Isolated colonocytes were incubated for 15 min at 25°C with 2 μM carboxy SNARF-1 acetoxymethylester acetate (SNARF-1/AM acetate, Molecular Probes Inc., Eugene, OR) in 'Na medium' (containing in millimolar: 130 NaCl, 5 KCl, 1 MgSO₄, 2 CaCl₂, 20 HEPES, 25 mannose, pH 7.4). After dye loading, 50-100 μl aliquots of cells were loaded into a chamber (Montrose, Friedrich, and Murer, 1987). Colonocytes were allowed to attach for 5-10 min to the glass coverslip which formed the base of the chamber. The chamber was then mounted on the stage of a Zeiss Axiovert microscope and perfused continuously throughout the duration of the experiment. Cells in the chamber were maintained at 35-37°C by heating the chamber and objective lens via jacketed water circulation. Cells were visualized with a 100× (Zeiss Neofluar, 1.3 NA) objective, and fluorescence excited with a 75 W Xenon lamp. SNARF-1 fluorescent emission was measured at 590-610 nm in response to alternating excitation wavelengths of 505 ± 13 nm and 575 ± 7 nm. Excitation light was attenuated with a neutral density filter (10% transmitting, Omega Optical), and limited by a shutter (Ludl Electronics Products, Hawthorne, NY) which exposed the sample to excitation light only during data collection. Under these conditions, photobleaching was negligible (data not shown). Fluorescence was detected with a Hamamatsu intensified CCD camera (model C2400-97), and data collected as four frame averages (128 ms/image) which were digitized by an image processor (Perceptics, Knoxville, TN). The 575/505 fluorescence ratio was used as an indicator of pH, and was calibrated versus pH using 10 μM nigericin (Molecular Probes Inc.) and 120 mM K⁺ solutions as described previously (Thomas, Bushbaum, Zimniak, and Racker, 1979; Montrose et al., 1987; Watson, Levine, Donowitz, and Montrose, 1991). Although SNARF-1 was originally designed for use as a dye for emission ratioing applications (Bassnett et al., 1990; Buckler and Vaughan-Jones, 1990), we find that excitation ratioing is also practical. As shown in Fig. 1, a calibration curve of 575/505 fluorescence ratio versus pH was used to determine pH values from pH 6.0 to pH 8.1. In each experiment, up to 20 cells in the camera field were selected for simultaneous, real-time analysis. Each selected cell was analyzed separately during the experiment from a region of the image equal to 7.6 μm² (116 pixels). Average pixel intensity in each region was used to measure cellular response. Unless otherwise stated, results from a single experiment are presented as average values from all cells selected for real-time analysis. Background values (camera dark level) were determined before each analysis and were subtracted from fluorescence values of SNARF-1 loaded cells before calculation of the fluorescence ratio. Cellular (and system) autofluorescence was negligible (<2% of fluorescent signal) under these experimental conditions, so data were not corrected for autofluorescence.

**Perfusate Solutions**

To study pH under Na⁺ free conditions, two general types of perfusate solution were used: TMA medium which was made by mol:mol substitution of tetramethylammonium chloride (TMACl) for all sodium chloride in the Na medium and TMA SCFA media which was made by mol:mol substitution of a SCFA for chloride in the TMA medium (TMA salts were made in the
laboratory from TMAOH and the appropriate carboxylic acid). In most experiments, propionate was used as the test SCFA. The [SCFA] was 65 mM unless otherwise stated. The pH of perfusate solutions was adjusted to pH 7.40 before each experiment unless otherwise stated. Other simple changes from the basic solutions described above will be stated in the text describing specific experiments. Chemicals were purchased from Fluka Chemical Co. (Ronkonkoma, NY) or Sigma Chemical Co., unless indicated otherwise.

RESULTS

Cells were selected for study based on (a) the presence of a visible brush border membrane and/or inclusion of cells in an epithelial layer of cells and (b) cytosolic loading with SNARF-1 after exposure to carboxy SNARF-1/AM acetate (see Methods). Fig. 2 shows transmitted light images of a dye-loaded crypt fragment (Fig. 2 A) and the cytosolic SNARF-1 fluorescence of these cells before (Fig. 2 B) and after (Fig. 2 C) addition of 10 μM digitonin. As shown qualitatively in the figure, 5 min incubation with digitonin released 91 ± 2% of the dye (mean ± SEM, n = 4 preparations) and did not unmask any intracellular sites of particulate fluorescence.

Experiments documented that colonocytes were viable using propidium iodide (PI) to assay plasma membrane integrity. PI enters cells with increased membrane permeability (e.g., leaky or dead cells) and stains DNA with resultant bright nuclear fluorescence (Montrose, Condrau, and Murer, 1989). Fig. 2 D shows a transmitted light image of an isolated crypt loaded with SNARF-1, and superfused with medium containing 5 μM PI. Images of PI fluorescence are shown before (Fig. 2 E) and after (Fig. 2 F) exposure to digitonin. As shown, the majority of colonocytes in the preparation are viable (i.e., exclude PI under normal conditions). Results in Fig. 2 suggested that freshly isolated colonocytes loaded with SNARF-1 could be a reliable model for the study of pH_i in viable cells.
Effect of Propionate on Colonocyte pH

Similar to other epithelial and nonepithelial cell types (Nakhoul and Boron, 1988; Grinstein, Goetz, Furuya, Rothstein, and Gelfand, 1984; Montrose, Knoblauch, and Muter, 1988; Feldman, Ziyadeh, Mills, Booz, and Kleinzeller, 1989), mouse colonocytes acidify rapidly after SCFA exposure (Fig. 3 A, ab). In many cell types, this has commonly been attributed to nonionic diffusion of the protonated SCFA (Roos and Boron, 1981), although the presence of nonionic diffusion across colonocyte membranes has recently been debated (Rajendran and Binder, 1994). After the acidification induced by 130 mM propionate, colonocytes demonstrated both Na⁺-independent (Fig. 3 A, bc) and Na⁺-dependent (Fig. 3 A, cd) pHᵢ alkalinization. In six preparations, pHᵢ recovery was measured over a range of pHᵢ 6.7–7.0 and the initial rate of Na⁺-independent pHᵢ recovery was found to be 65 ± 8% (mean ± SEM,
n = 44 cells) of the total pHᵢ alkalinization rate following the subsequent addition of 140 mM Na⁺ to the perfusate. In the population, only 9% of the cells (4/44) had Na⁺-independent pHᵢ recovery which was < 12% of the total alkalinization rate, suggesting that Na⁺-independent pHᵢ recovery was expressed at a high level in the majority of isolated colonic crypt cells.

As shown qualitatively in Fig. 3 B, colonocytes started with an initial pHᵢ in Na medium of 7.43 ± 0.01 (mean ± SEM, n = 26 cells). After removal of Na⁺ (substitution with TMA⁺) in Cl⁻ containing medium, cells slowly acidified to pH 7.17 ± 0.12 (n = 84) over 3–5 min. After exposure to 65 mM propionate, the Na⁺-independent pHᵢ alkalinization mechanism allowed colonocytes to recover pH from an average acidified pHᵢ of 6.64 ± 0.03 to a steady state pHᵢ of 7.01 ± 0.02 (mean ± SEM, n = 55 cells). Upon subsequent removal of propionate from the perfusates, pHᵢ rapidly alkalinized (Fig. 3 B, cd) and then slowly returned to the resting pHᵢ observed
before propionate exposure (Fig. 3 B, de). Because the alkalinization raised \( \text{pH}_i \) to a level higher than starting \( \text{pH}_i \) in TMA medium, we will use the term \( \text{pH}_i \) overshoot to describe the phenomenon.

Propionate and all physiologic SCFAs exist in two titratable forms: nonionized (protonated) and ionized (anionic). Experiments were designed to test which molecular form of propionate was responsible for the observed acidification and \( \text{Na}^+ \)-independent \( \text{pH}_i \) recovery. In the first experiment, the concentration of nonionized propionate in the medium was held constant whereas the concentration of propionate anion was varied threefold. The concentration of nonionized propionate was fixed at 0.65 mM by adjusting the medium \( \text{pH} \) and total propionate concentration simultaneously according to the Henderson-Hasselbach equation (\( pK_a = 4.88 \)). As shown qualitatively in Fig. 4 A, the acidification due to 21.7 mM propionate at \( \text{pH} 6.9 \) (0.48 ± 0.03 \( \text{pH} \) units, mean ± SEM, \( n = 20 \) cells) was not significantly different in a paired comparison with the acidification caused by 65 mM propionate at \( \text{pH} 7.4 \).
In contrast, despite acidification to a similar extent, the rate of pH$_i$ recovery was greatly diminished ($P < 0.001$) when the anion concentration was lowered (0.15 ± 0.02 pH units/min at medium pH 7.4 vs 0.005 ± 0.013 pH units/min at medium pH 6.9). These results imply that Na$^+$-independent pH$_i$ recovery was inhibited either by reduction of the extracellular propionate anion concentration and/or acidification of the medium.

To help discriminate between these possibilities a second experimental series varied medium pH from 6.9 to 7.4 and compared the response to 65 mM total propionate. In these conditions, the concentration of propionate anion was similar (64.35 mM at pH 7.4 and 63.05 mM at pH 6.9), but the concentration of nonionized propionate was threefold higher at pH 6.9 (1.95 mM) than at pH 7.4 (0.65 mM). As shown qualitatively in Fig. 4B, acidification at pH 6.9 (0.64 ± 0.03 pH units, mean ± SEM, $n = 20$ cells) was significantly greater ($P < 0.001$) vs pH 7.4 (0.48 ± 0.02 pH units). In this experiment, the rate of Na$^+$-independent pH$_i$ recovery at pH 6.9 was greater than at pH 7.4 (0.22 ± 0.02 pH/min and 0.16 ± 0.02 pH/min, respectively; $P < 0.05$). The latter result clarifies that acidifying media to pH 6.9 does not eliminate Na$^+$-independent pH$_i$ recovery, however, significant differences in pH$_i$ between conditions weakens rigorous comparison of rates in Fig. 4B (because the rate of Na$^+$-independent pH$_i$ recovery may be pH$_i$ sensitive).

Combined with the results of Fig. 4A, we conclude that (a) the extent of cellular acidification is selectively affected by changes in the concentration of the nonionized form whereas (b) the Na$^+$-independent pH$_i$ recovery is inhibited by lowering extracellular propionate anion and/or extracellular pH. Results support the working hypothesis that nonionic diffusion causes cellular acidification, but further experiments must test whether SCFA uptake is directly responsible for Na$^+$-independent pH$_i$ alkalinization.

**Basis for the pH$_i$ Overshoot**

It was hypothesized that the pH$_i$ overshoot was due to an increased amount of propionate anion taken up by colonocytes during Na$^+$-independent pH$_i$ recovery. Net SCFA uptake is predicted during pH$_i$ alkalinization due to (a) redistribution of molecular forms via SCFA nonionic diffusion or (b) direct uptake via a SCFA anion transporter. Upon removal of propionate, rapid redistribution of molecular forms would support rapid efflux of all propionate forms via nonionic diffusion. This would cause an overshoot alkalinization.

One test of the hypothesis was to demonstrate that appearance of an overshoot was not a direct consequence of cellular acidification. Exposure of colonocytes to propionate for 30 s allowed full acidification, but there was no detectable pH$_i$ recovery and no pH$_i$ overshoot after propionate removal (Fig. 5, bc). More prolonged exposure to propionate was required to observe significant Na$^+$-independent pH$_i$ recovery (Fig. 5, ef) and the pH$_i$ overshoot (Fig. 5, fg). This suggested that acidification alone was not sufficient to generate an overshoot.

It was also possible to observe a decrease in overshoot after a prolonged acidification in which Na$^+$-independent pH$_i$ recovery had been inhibited by medium acidification coincident with decreased propionate anion concentration (Fig. 4A). To quantify the magnitude of the overshoot alkalinization, the pH$_i$ overshoot was
defined as the maximal \( \Delta pHi \) caused by propionate removal minus the \( \Delta pHi \) of initial acidification (in Fig. 5 B, overshoot = cd - ab). When the Na\(^+\)-independent pHi recovery was inhibited by reduction of extracellular propionate anion, the overshoot was also significantly reduced (65 mM/pH 7.4, 0.32 ± 0.05 pH unit overshoot; vs 21.7 mM/pH 6.9, 0.04 ± 0.03 pH unit overshoot, n = 20 cells, \( P < 0.001 \)).

Results in Fig. 4 had shown that flux of nonionized propionate (putative nonionic diffusion) was responsible for cellular acidification. Further experiments tested whether the same process was able to account for the alkalization of pHi after propionate removal. Experiments compared estimates of intrinsic buffering capacity generated from acidification upon propionate addition vs alkalization when propionate was removed after 3 min exposure. Cells were exposed to variable concentrations of propionate (8–32 mM) to vary the extent of acidification, and results grouped according to the median pH of the pH excursion (all \( \Delta pHi < 0.3 \)). As shown in Fig. 6, the values from the two methods overlapped extensively. To calculate the buffering capacity from propionate removal, the intracellular [propionate] is estimated assuming nonionic diffusion has enforced transmembrane equilibration of the nonionized form, and that all propionate efflux is in the nonionized form (Roos and Boron, 1981). Combined with the observation that the nonionized form is responsible for acidification, results suggest that the process which causes both acidification and the pHi overshoot is nonionic diffusion (or a protein-mediated flux of nonionized propionate which involves no other titratable acid/base equivalents).

The observed pHi dependence of buffering capacity was unusual, because most cells have increased buffering capacity at lower pH. To question whether it was the propionate or colonocytes which gave unusual results, experiments directly compared buffering capacity estimated by addition of propionate vs ammonium (a commonly applied weak base), in the same cells. As shown in Fig. 6, values from the weak base (a) had a qualitatively similar pHi-dependence as other mammalian cells, but (b) were different from propionate. Importantly, if transmembrane flux of propionate anion was compromising the buffering capacity measurement, the pHi change upon propionate addition would be smaller, and the resultant calculated buffering capacity larger, than the real value. Thus, corruption of measurements by
propionate anion flux cannot readily explain differences between ammonium and propionate. As discussed later (see Discussion), the buffering capacity sensed by propionate was used in further experiments as the appropriate value for calculating proton fluxes stimulated during propionate exposure.

Concentration Dependency of the Cellular Response to Propionate

The magnitude of acidification, initial Na\(^+\)-independent pH\(_i\) recovery rate and pH\(_i\) overshoot were each plotted independently vs concentration of propionate applied. As shown qualitatively in Fig. 7, all three variables are dependent on the applied [propionate] over the range of 8–130 mM. In the following figures, results were compiled from 38 colonocytes in four individual experiments performed as in Fig. 7.
The acidification was measured as the maximal difference between the pHi before versus after propionate exposure (e.g., $\Delta$pH = $ab$ in Fig. 3). As shown in Fig. 8 A, the magnitude of acidification showed a positive linear relationship ($r = 0.99$) versus the amount of propionate applied. When the $\Delta$pH of acidification was transformed into a net H$^+$ increase ($\Delta$pH $\times \beta$, mM) to estimate net propionate uptake, results were observed to saturate at higher propionate concentrations (Fig. 8 B). The data were well fit by Michaelis-Menten kinetics with a $K_t$ (propionate) = 32 mM. Independent of mechanism, the result suggests that colonocytes can manifest a mechanism which rapidly limits the net propionate uptake.

The Na$^+$-independent pHi recovery rate, in response to the same range of propionate concentrations, was measured as the slope of initial linear phase of the pHi recovery (pH/min), and converted to a net H$^+$ efflux rate (mM/min) by multiplication with the total buffering capacity ($\beta_i + \beta_{net}$) at the initial pHi of the measurement. As shown in Fig. 9 A, data showed a tendency to saturation at high

$\beta$, at the appropriate pHi value was determined from propionate data in Fig. 6. $\beta_{prop}$ was estimated as 2.305 [intracellular propionate anion], as derived previously for other weak acids (Roos and Boron, 1981). The inclusion of this factor is only required for conditions in which a weak acid is available for transmembrane buffering during the entire measurement of a pH change. It is not required during estimation of pH changes caused by addition or removal of a weak acid because this is assumed to be a one-way reaction which does not contribute to cellular buffering (Roos and Boron, 1981).
[propionate], and sigmoidicity at low [propionate]. Data were poorly fit by the Michaelis-Menten equation, but were well fit by the Hill-equation (Kt = 41 mM with a best fit Hill coefficient = 2.1). These initial rates are complex, because (a) SCFA uptake has occurred before rate measurement (i.e., not zero trans SCFA conditions), (b) measured pH; changes are likely to involve both nonionic and ionic SCFA fluxes (see Discussion), and (c) pH; varies among tested SCFA concentrations (rates of Na'-independent pH; recovery may be pH;-sensitive). However, fluxes saturate versus the driving force for net uptake (energy in transmembrane propionate gradient), despite differences in driving force among tested SCFA concentrations.

Figure 9. Propionate concentration dependency of Na+-independent pH; recovery. (A) Initial rate of H+ efflux (measured during the initial 2 min of Na+-independent pH; recovery) saturates vs medium propionate concentration. Results were expressed as mean ± SEM (n = 38 cells). Two curve fits are presented in which data are modeled to Michaelis-Menten kinetics (solid line; Kt = 132 mM) or the Hill equation (dotted line; Kt = 41 mM, Hill coefficient = 2.09). (B) Same initial H+ efflux rates also saturate versus transmembrane driving force. Driving force is shown in two scales of electrochemical potential (Δμ; prop) and estimated transmembrane propionate gradient ([prop]/[prop];). Concentration of propionate (in millimolar) is written above each point. Estimates of electrochemical potential assumed membrane potential did not contribute to driving force, because other experiments (Fig. 14) suggest that changes in membrane potential do not affect Na+-independent pH; recovery.

(Fig. 9 B). We conclude that (a) transport saturates independent of SCFA driving force, but (b) because of different driving forces at low [propionate] the sigmoidicity of activation kinetics should not be used to infer kinetic mechanisms of propionate transport.

Because results suggested that the pH; overshoot was an indirect measure of propionate uptake, we appraised this as an independent measure of the Na+-independent pH; recovery. As shown in Fig. 10 A, the linear correlation between the magnitude of pH; overshoot and net H+ efflux was significant (r = 0.97). To estimate
the H⁺ efflux (in millimolar) during a pHᵢ overshoot, the overshoot ΔpH was multiplied by intrinsic buffer capacity (βᵢ) of colonocytes. As shown in Fig. 10 B, the amount of net proton efflux during an overshoot shows a similar dependency on propionate concentration as the direct measure of Na⁺-independent pHᵢ recovery in Fig. 9. The fit to the Hill equation results in a similar Kᵢ (37 mM) and a slightly smaller Hill coefficient (nH = 1.6). Overall, results in Figs. 9 and 10 (a) corroborate the suggestion of sigmoidicity and saturability in propionate activation kinetics, and (b) support the use of SNARF-1 for quantitative measurements at both acidic and alkaline pHᵢ.

**Potential Substrates and Inhibitors of Na⁺-independent pHᵢ Recovery**

To further test whether SCFAs directly mediate Na⁺-independent pHᵢ recovery, experiments tested for structural specificity in the putative SCFA transporter. The three SCFAs which are most abundant in the colonic lumen (acetate, propionate and n-butyrate) had qualitatively the same effect on pHᵢ of colonocytes (Fig. 11). When exposed to these SCFAs, colonocytes acidified promptly, and then a Na⁺-independent pHᵢ recovery was observed to alkalinize pHᵢ to ~ pH 7.0. Upon SCFA removal, immediate alkalinization and a pHᵢ overshoot were always observed. As shown in Fig. 11 B, similar results were observed with n-butyrate and iso-butyrate, despite the large difference in metabolism of these two naturally occurring SCFAs (Weigand, Young,
and McGillard, 1975; Bugaut, 1987). This suggests that pH changes are due to transport, not metabolism, of the SCFAs.

Several weak acids did not elicit Na⁺-independent pHᵢ recovery. As shown in Fig. 11, A and C, the weak acids formate and lactate (2-hydroxy propionate) caused intracellular acidification, but Na⁺-independent pHᵢ recovery was not observed. Results from these naturally occurring weak acids are compiled in Table I, which establishes that the similar acidification caused by all compounds does not correlate with their diverse potency to elicit Na⁺-independent pHᵢ recovery. This demonstrates
that the Na⁺-independent pHᵢ recovery mechanism has a defined substrate specificity that is satisfied by the SCFAs found most abundantly in the colonic lumen.

A modified SCFA, 3-mercaptopropionate, has been used previously to inhibit SCFA transport in colon and gallbladder (Stein, Schroder, Milovic, and Caspary, 1995; Holtug, 1993). As shown qualitatively in Fig. 12A, this compound acidified colonocytes as effectively as propionate (113 ± 4% of propionate response, n = 18 cells), but Na⁺-independent pHᵢ recovery and pHᵢ overshoot were slow (14 ± 3% and 16 ± 5% of propionate response, P < 0.001 respectively, n = 18 cells). This is the first demonstration that 3-mercaptopropionate affects cell pH, which complicates the previous interpretation of its action as a direct inhibitor. When we tested the efficacy of 3-mercaptopropionate as an inhibitor, there was no difference between preincubation with 10 mM 3-mercaptopropionate versus 10 mM propionate: both elicited a similar apparent decrease in Na⁺-independent pHᵢ recovery (pH/min) observed during subsequent addition of 65 mM propionate (data not shown). The apparent inhibition in both cases is likely to be due to increased buffering capacity in the presence of the SCFA. Malonate, a dicarboxylate, did not affect colonocyte pHᵢ (Fig. 12 B), suggesting that malonate could not permeate colonocytes via any mechanism. In addition, neither 10 mM butanol nor 10 mM ethanol affected colonocyte pHᵢ (data not shown).

To examine sensitivity of the SCFA transporter to known transport inhibitors, several drugs were tested for their effect on Na⁺-independent pHᵢ recovery. Stilbene derivatives are known as effective inhibitors of anion transport (Poole and Halestrap, 1993), and are known to inhibit SCFA⁻/Cl⁻ exchange (Rajendran and Binder, 1994),

| Acidification | Recovery rate | n |
|---------------|---------------|---|
| Formate       | 0.56 ± 0.02   | -0.006 ± 0.011 | (20) |
| Acetate       | 0.67 ± 0.02   | 0.13 ± 0.01    | (25) |
| Propionate    | 0.64 ± 0.02   | 0.15 ± 0.01    | (41) |
| Lactate       | 0.50 ± 0.03   | -0.05 ± 0.01   | (8)  |
| n-butyrate    | 0.57 ± 0.03   | 0.15 ± 0.01    | (13) |
| Iso-butyrate  | 0.53 ± 0.04   | 0.15 ± 0.00    | (5)  |

*SNARF-1 loaded colonocytes were exposed to 65 mM of the indicated weak acid as a TMA salt, and the subsequent changes in pHᵢ measured. The table presents the maximal acidification observed directly after exposure to the acid as a ΔpH value. Because the extent of acidification was similar in all cases (and the amount of applied weak acid was identical), the Na⁺-independent pHᵢ recovery rate was calculated in units of pH/min. Results presented as mean ± SEM are compiled from six experiments and the total number of individual cells studied under each condition is given in the table (n). Using a two-tailed unpaired t test, results, in each column were compared to the effect of propionate. Unless noted (𝑃 < 0.001), values were not significantly different from the effect of propionate (𝑃 > 0.05).
SCFA-stimulated volume regulation (Rowe, Blackmon, and Montrose, 1993), and some SCFA⁻/HCO₃⁻ exchange reactions (Harig et al., 1991; Reynolds et al., 1993). Two stilbenes were tested at 1 mM; 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS). An inhibitor for H⁺/monocarboxylate cotransporter, α-cyano-4-hydroxycinnamate (CnCN) (Poole and Halestrap, 1993; Rosenberg et al., 1993), was used at 4 mM. The organic anion exchange inhibitor probenecid was used at 1 mM (Guggino and Guggino, 1989). Colonocytes were first exposed to 65 mM propionate in Na⁺ free medium as the control, then the same cells were exposed to an inhibitor for 3 min followed by 65 mM propionate solution which also contained the inhibitor. The rate of

\[
\text{Na}^+ \text{-independent } pH_i \text{ recovery was calculated from the initial 2 min of the } pH_i \text{ recovery and expressed as } \Delta pH/\text{min. The effects of inhibitors were estimated as percent of control } pH_i \text{ recovery rate from the same cells. Data collected from 25-45 cells for each inhibitor are presented in Fig. 13. The results demonstrated that none of the four inhibitors significantly suppressed the } pH_i \text{ recovery rate. In addition (data not shown), no drug affected base line } pH_i \text{ before propionate exposure, or affected the acidification caused by propionate. Results suggest that the } Na^+ \text{-independent } pH_i \text{ recovery of mouse colonocytes is unlikely to be mediated by previously described SCFA transporters.}
\]

**Figure 12.** Effect of nonphysiologic weak acids on intracellular pH of colonocytes. (A) 3-mercaptopropionate (65 mM) acidified colonocytes but did not effectively activate Na⁺-independent pHᵢ recovery or cause pHᵢ overshoot when removed from the perfusate. Average results from six colonocytes in a single experiment are presented. Identical results were obtained from three experiments. (B) Malonate (65 mM) did not acidify colonocytes. Average results from eight colonocytes in a single experiment are presented. Identical results were obtained from three experiments. (Open bars) TMACI medium (pH 7.4).
Independence of Na\(^+\)-independent pH\(_i\) Recovery from Changes in Inorganic Ions and Membrane Potential

Experiments tested whether Na\(^+\)-independent pH\(_i\) recovery was affected by alterations in medium K\(^+\) concentration. Fig. 14A demonstrates that Na\(^+\)-independent pH\(_i\) recovery still occurs in K\(^+\)-free conditions, with no effect of rapidly switching cells into high potassium medium (plus valinomycin). These experiments show that the pH\(_i\) recovery mechanism does not require K\(^+\), and also suggest that changes in membrane potential may not affect Na\(^+\)-independent pH\(_i\) recovery. Effects of membrane potential are tested more rigorously in Fig. 14B, in which changes in medium K\(^+\) (plus valinomycin) are shown to have no effect on pH\(_i\) until after addition of the electrogenic protonophore, FCCP. Changes in pH\(_i\) in the presence of FCCP act as a positive control demonstrating changes in membrane potential, and our

![Figure 13. Effects of known anion transport inhibitors on the SCFA transporter.](image)

ability to detect changes in pH\(_i\) when electrogenic proton fluxes are present. These experiments establish that Na\(^+\)-independent pH\(_i\) recovery is electroneutral, and eliminate the possibility that a "SCFA-requiring" H\(^+\), K\(^+\)-ATPase or electrogenic H\(^+\)-ATPase (Engelhardt, Burmester, Hansen, Becker, and Rechkemmer, 1993) was responsible for Na\(^+\)-independent pH\(_i\) recovery.

Experiments also tested whether Ca\(^{2+}\)/SCFA\(^-\) or Mg\(^{2+}\)/SCFA\(^-\) cotransport mediated the Na\(^+\)-independent pH\(_i\) recovery. Isolated colonocytes were exposed to 65 mM propionate in the presence of 0 and 10 mM Ca\(^{2+}\) or 0 and 10 mM Mg\(^{2+}\). The results showed neither Ca\(^{2+}\) nor Mg\(^{2+}\) affected the rate of Na\(^+\)-independent pH\(_i\) recovery (data not shown).

Because electroneutral SCFA anion transport through SCFA\(^-\)/Cl\(^-\) exchange has been suggested (Petersen et al., 1981; Binder and Metha, 1989; Binder and Rajendran, 1993), we directly examined the possible role of Cl\(^-\) on the electroneutral
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A

\[ [K^+] : \quad 0 \text{ mM} \quad \frame{135 \text{ mM (+} 10\mu\text{M Val)} \]

\begin{align*}
\text{pH} \quad & 8.6 \quad 8.4 \quad 8.2 \quad 7.8 \quad 7.4 \quad 7.0 \quad 6.6 \quad 6.2 \\
\hline
\end{align*}

3 min

B

\[ [K^+] \text{ mM:} \quad 5 \quad 135 \quad 5 \quad 135 \quad 5 \]

\begin{align*}
\text{pH} \quad & 7.3 \quad 7.2 \quad 7.1 \quad 7.0 \quad 6.9 \\
\hline
\end{align*}

3 min

**FIGURE 14.** Effect of medium K⁺ and membrane potential on the SCFA transporter. (A) Colonocytes were preincubated in K⁺-free medium for 30 min before experiments and initially perfused in K⁺-free TMACl medium (open bar). Adding 135 mM TMAprop in a K⁺-free medium (black bar) produced acidification and Na⁺-independent pHᵢ recovery in colonocytes. Replacement of K⁺-free TMAprop with 135 mM potassium propionate (Kprop) plus 10 μM valinomycin (gray bar) did not affect Na⁺-independent pHᵢ recovery. The pHᵢ trace presents average of eight colonocytes in a single experiment. Identical results were observed in four experiments. (B) Experiments compare response to changes in medium K⁺ before and after exposure to the electrogenic protonophore, FCCP (10 μM). Cells were initially perfused in TMACl medium (with 5 mM KCl), then exposed to different isomotic media, all containing 65 mM propionate and 10 μM valinomycin. Variations in medium K⁺ were made by switching between Kprop (135 mM K⁺) and the usual formulation of TMAprop medium (5 mM K⁺).
SCFA transport activity observed. Gluconate was used to replace Cl⁻ mol:mol in TMA medium and propionate-containing medium. Isolated colonocytes were preincubated in Cl⁻-free sodium gluconate solution for 30 min. As shown in Fig. 15 A, pHᵢ recovery was similar in both the presence and absence of Cl⁻. Similarly, changes in the Cl⁻ gradient during a single acidification did not change intracellular pH (Fig. 15 B).

**Figure 15.** Effect of changes in transmembrane Cl⁻ gradient on the SCFA transporter. Colonocytes were preincubated in Cl⁻-free medium (gluc = Cl⁻ replaced mol:mol with gluconate) for 30 min before experiments and initially perfused in the same medium. (A) Cells were transiently exposed to a Cl⁻-free TMAprop (gluc/prop = 1:1 mixture of 130 mM TMAgluc and TMAprop in Cl⁻-free medium) perfusate. The Cl⁻-free condition did not affect either Na⁺-independent pHᵢ recovery or pHᵢ overshoot. Chloride (65 mM) was then restored to perfusate (Cl/gluc = 1:1 mixture of 130 mM TMACl and TMAgluc) and cells acidified by exposure to Cl⁻-containing TMAprop (Cl/prop = 1:1 mixture of 130 mM TMACl and TMAprop) as a control. The pHᵢ trace presents average response of 14 colonocytes in a single experiment. (B) During the steady state pHᵢ, after Na⁺-independent pHᵢ recovery, switching perfusion between Cl⁻-free (gluc/prop) and Cl⁻-containing (Cl/prop) TMAprop did not affect steady state. The pHᵢ trace presents average of 10 colonocytes in a single experiment. Similar results were observed from three (A) or four (B) experiments.
DISCUSSION

In this work, we introduce a new system for studying the effect of SCFAs on colonocytes. Mouse colonocytes have been isolated in a preparation that allows study of single viable cells within an epithelial layer. The cells maintain a polarized epithelial morphology (visible brush border, attachment to adjacent cells in a structure resembling native colonic crypts), but colonocytes can be studied free of submucosal structures. Attachment of cells to a glass coverslip (forming the base of a microscope chamber) allows continuous perfusion during visualization at high magnification. The preparation does not allow separation of events at the apical versus basolateral domain, but may be helpful for the definition of transporters and regulatory events which have been difficult to observe at the level of whole tissue or isolated membrane vesicles.

In the current work, we have used colonocytes isolated from the entire colon, and restricted our functional analysis to events which occur in virtually all viable colonocytes. Similar to most mammals, mice have high concentrations of SCFAs in the colonic lumen (Hoverstad et al., 1985; Hoverstad and Midtvedt, 1986). Our results demonstrate that SCFAs elicit acidification in all colonocytes studied (data not shown), and significant Na+-independent pHi recovery in >90% of the colonocytes studied. This suggests that our observations represent the response of the majority of crypt colonocytes in the mouse colon. The expression of Na+-independent pHi recovery in cells throughout the colon may indicate the essential nature of this function for colonocyte survival.

After exposure of colonocytes to SCFAs, the first detectable event is a rapid cellular acidification of ~0.5 pH units. Four possibilities were considered as possible mechanisms to explain the rapid SCFA-induced intracellular acidification: (a) non-ionic diffusion of SCFAs, (b) SCFA^−/H^+ cotransport, (c) SCFA^−/HCO_3^− exchange, and (d) cellular metabolism. We observed that (a) a specific increase in nonionized propionate concentration resulted in greater acidification (Fig. 4 B), (b) acidification was not affected by specific changes in propionate anion concentration (Fig. 4 A), (c) inhibitors of H^+/monocarboxylate cotransport or most SCFA^−/HCO_3^− exchange reactions (CnCN, DIDS) did not inhibit acidification (data not shown), and (d) acidification was similar for SCFAs with widely varying metabolism (e.g., n-butyrate vs iso-butyrate; Table I). These results support simple diffusion of nonionized SCFAs as the primary mechanism mediating intracellular acidification.

A confounding observation was that the propionate uptake during acidification saturated vs propionate concentration. This is not predicted for nonionic diffusion, but has been observed previously for the acidification of renal proximal tubules by lactate (Siebens and Boron, 1987). This suggests either that nonionic uptake is protein mediated, that some unidentified condition (e.g., a pH microdomain near the plasma membrane; Jackson et al., 1978) limits the uptake of propionate by nonionic diffusion, or that Na^+-independent pHi recovery compromises the maximum extent of acidification. The last alternative is unlikely, since the extent of acidification by lactate, formate and 3-mercaptopropionate (which only poorly satisfy the Na^+-independent alkalinization mechanism) was indistinguishable from propio-
nate. Further, it is clear that nonionized SCFAs are highly permeable to lipid bilayer membranes in vitro (Walter and Gutknecht, 1984), and nonionic diffusion of SCFAs has been supported as a major mechanism for intestinal SCFA flux in studies of jejunum and colon (Naupert and Rommel, 1975; Schmmit et al., 1976; Jackson et al., 1978; Ronnau et al., 1989).

Estimates of buffering capacity were qualitatively and quantitatively different when comparing results from propionate versus ammonium addition. Evidence suggests that the difference is neither artifactual, nor due to problems with propionate measurements. Both buffering capacity estimates were from the same cells, therefore differences are not explained by cell–cell variability. Initial acidifications elicited by a broad range of monocarboxylates were similar (Table I), suggesting results were not due to a peculiarity of propionate. Further, high NH₄ permeability, but not high propionate anion permeability, could explain results in Fig. 6 because high ionic permeability (relative to nonionic permeability) will diminish pH excursions and falsely raise buffering capacity estimates. Differences may also occur between use of a weak acid vs a weak base. Cytosolic pH excursions from nonionic diffusion may be diminished by accumulation of weak bases in intracellular acidic spaces (e.g., lysosomes), and/or increased by relative exclusion of weak acids from acidic organelles. We do not have sufficient information to discriminate among possible mechanisms which could explain results, and the explanation for the difference remains obscure.

Faced with disparate estimates of buffering capacity, we had to choose appropriate values to use for calculating net proton flux. Selection of the propionate values was not arbitrary. Assuming Fig. 6 is accurate, results indicate that cytosolic resistance to pH change is low when propionate is added to colonocytes (for unknown reasons). The propionate values were used because is seemed reasonable that a similar cytosolic resistance to pH change should also rule during the continued presence of propionate (i.e., during Na⁺-independent pHᵢ recovery). Experimental evidence suggests this choice was correct. Similar propionate activation kinetics were estimated from initial proton efflux rates (Fig. 9A) vs proton efflux estimated from the overshoot (Fig. 10B); even though these two data sets use buffering capacity values from an acidic or alkaline pHᵢ range, respectively. This internal consistency is unlikely to be observed if the pHᵢ dependence of buffering capacity was incorrect.

After the initial cellular acidification caused by uptake of nonionized SCFAs, colonocytes alkalinized by both Na⁺-dependent and Na⁺-independent pHᵢ recovery mechanisms. The Na⁺-dependent recovery mechanism was not studied further, but is likely to be due to activation of plasma membrane Na⁺/H⁺ exchange and/or Na⁺/monocarboxylate cotransport. Acidification due to SCFAs is known to activate Na⁺/H⁺ exchangers in cells derived from colon (Holtug, 1989; Gabel et al., 1991; Diener, Helme-Kolb, Murer, and Scharrer, 1995; Rowe et al., 1994) as well as a number of other epithelial cells (Petersen et al., 1981; Siebens and Boron, 1987; Nakhoul and Boron, 1988). This is often due to allosteric activation of Na⁺/H⁺ exchange by intracellular protons (Aronson, Nee, and Suhm, 1982). The more novel observation was of a Na⁺-independent mechanism allowing colonocytes to recover from SCFA acidification, therefore, this process was studied in detail.

Experiments suggest that Na⁺-independent pHᵢ recovery could be detected either
as a pH recovery during SCFA exposure, or as an alkaline overshoot of pH when accumulated SCFA effluxed from cells. Measurements of intrinsic buffering capacity suggested that upon SCFA removal, the total alkalinization was generated by loss of cellular SCFA via nonionic efflux. However, the overshoot should not be taken as diagnostic of a SCFA-dependent pH recovery mechanism, because nonionic diffusion will also lead to intracellular SCFA accumulation when cells alkalinize (because of the redistribution of molecular forms). Several protocols suggested that the pH overshoot was a (direct or indirect) consequence of Na+-independent pH recovery. Overshoots were not observed (or were greatly diminished) if Na+-independent pH recovery was limited by (a) brief pulses of propionate (Fig. 5); (b) coincident lowering of propionate concentration and medium pH (Fig. 4A); or (c) exposure to weak acids which did not demonstrate Na+-independent pH recovery (e.g., lactate, 3-mercaptopropionate or formate). Further, estimates of the propionate activation kinetics were similar in measurements of pH recovery rates (Fig. 9) or the magnitude of the associated overshoot (Fig. 10). These results suggest that pH overshoots are valid to quantify Na+-independent pH recovery. Although it is important to rigorously control the time of exposure to SCFAs when comparing overshoots between conditions, a technical advantage of measuring overshoots is that the SNARF-1 dye is more responsive in the alkaline range.

As described below, evidence suggests that Na+-independent pH recovery was mediated by a SCFA transport process in the plasma membrane, and not a SCFA-independent pH alkalization mechanism stimulated by cellular acidification. First, the Na+-independent pH recovery demonstrated saturation by extracellular SCFA, independent of driving force for net SCFA uptake (Figs. 9 and 10). Most importantly, Na+-independent pH recovery demonstrated substrate specificity for naturally occurring SCFAs; unrelated to differences in (a) metabolism, or (b) hydrophobicity among tested monocarboxylic acids. Since initial acidification was the same among all tested monocarboxylates (Table I), results (a) demonstrate independence of the acidification mechanism from activation of Na+-independent pH recovery, and (b) suggest that in no case was acidification masking rapid Na+-dependent pH recovery (even during slow acidification by formate or lactate). We conclude that several weak acids were identified which acidified cells, but did not activate the pH recovery mechanism. These results are difficult to reconcile with a model in which SCFA anions are impermeant, and Na+-independent pH recovery is mediated by a SCFA-independent mechanism. Results suggest that a saturable, substrate-specific SCFA transporter mediates the Na+-independent pH recovery.

It is simple to envision how uptake of SCFA anions could lead to cellular alkalinization. Rapid acidification via nonionic diffusion establishes an equilibrium in which the molecular forms of a SCFA (ionized and nonionized) are at equilibrium with the cytosolic pH and the transmembrane pH gradient (Roos and Boron, 1981). A slow uptake of SCFA anions via the Na+-independent mechanism under study will disturb this equilibrium and drive the intracellular chemical equilibrium towards net formation of nonionized SCFA. Even a small increase in nonionized SCFA concentration will drive net efflux of this acid equivalent, because the concentration of this form rapidly equilibrates across the membrane via nonionic diffusion. The same general mechanism (of rapid nonionic diffusion compromised by ionic uptake) has
been frequently invoked to explain the transient alkalinization from weak base addition (Roos and Boron, 1981). This implies that initial rates of $pH_i$ change which we measure are complex; reflecting both ionic uptake and subsequent reequilibration of the nonionized form. For this reason, activation kinetics can not be used to readily determine mechanism of SCFA transport. However these kinetics may be useful for appraising the physiologic activation and saturation of transport by extracellular SCFA, since similar transport conditions (including significant intracellular SCFA concentrations and acidified $pH_i$) are likely to occur in vivo. It should be noted that the alkalinizing effect of SCFA anion uptake will not occur if the anion is transported in exchange for alternative anion which is a stronger proton acceptor (e.g., SCFA$^-$/HCO$_3^-$ exchanger) or cotransported with an cosubstrate which is a stronger proton donor (e.g., SCFA$^-$/H$^+$ cotransport). Sodium-dependent lactate and acetate cotransporters have been found to cause intracellular alkalinization of renal proximal tubule cells (Siebens and Boron, 1987; Nakhoul et al., 1988; Nakhoul and Boron, 1988). With the exception of the Na$^+$ dependence, the mechanism of intracellular alkalinization suggested in the present study is theoretically similar to these reported mechanisms.

The structural specificity of the Na$^+$-independent SCFA transporter has been partially determined. Acetate, propionate, $n$-butyrate, and iso-butyrate satisfied the transporter, but formate, lactate, 3-mercaptopropionate, and malonate did not. Results suggested that to satisfy the transporter, SCFAs must be composed of an aliphatic carbon chain with at least one methyl group. Addition of a second charged group (malonate) or a hydrophilic side group (lactate, 3-mercaptopropionate) disrupted transport. However, addition of a branched aliphatic chain (iso-butyrate = 2-methyl propionate) still supported transport. A similar substrate specificity has been reported for the SCFA/HCO$_3^-$ exchanger (Harig et al., 1991; Binder and Mehta, 1991).

Experiments partially characterized the Na$^+$-independent pH$_i$ recovery as electro-neutral, and not affected by changes in the transmembrane gradient of Cl$^-$ or K$^+$. Several known transport inhibitors had no effect on the Na$^+$-independent SCFA transporter. These results suggested that the observed transport is unlikely to be mediated via an (electrogenic) SCFA channel, or via previously described or suggested SCFA transporters. The Na$^+$/SCFA$^-$ cotransporter requires Na$^+$ (Nakhoul and Boron, 1988). SCFA$^-$/Cl$^-$ exchange requires Cl$^-$ and is DIDS-sensitive (Rajendran and Binder, 1994). SCFA$^-$/HCO$_3^-$ exchange can only lead to alkalinization if it mediates HCO$_3^-$ uptake and SCFA$^-$ efflux, however the Na$^+$-independent mechanism requires extracellular SCFA anions. The H$^+$/monocarboxylate transporter inhibited by CnCN is predicted to cause acidification, not alkalinization (Poole and Halestrap, 1993; Rosenberg et al., 1993) during SCFA uptake.

Our experiments have identified an electroneutral SCFA transport process in mouse colonocytes which is distinct from previously described transporters. The SCFA transporter demonstrates substrate specificity appropriate for transporting physiologic SCFAs, but further experiments will be needed to address the role of the transporter in transepithelial SCFA transport. The current work establishes the presence of a previously unanticipated mechanism of SCFA transport which can contribute to pH$_i$ regulation in > 90% of isolated mouse colonocytes.
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