An Na^+-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 (pH_i) 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 *iso*-butyrate in isosmotic Na^+-free media (substituted with tetramethylammonia), all colonocytes acidified rapidly and then >90% demonstrated a pH_i alkalinization (Na^+-independent pH_i recovery). Upon subsequent removal of the SCFA, pH_i alkalinized beyond the starting pH_i (a pH_i overshoot). Using propionate as a test SCFA, experiments demonstrate that the acidification and pH_i overshoot are explained by transmembrane influx and efflux of nonionized SCFA, respectively. The basis for the pH_i overshoot is shown to be accumulation of propionate during pH_i alkalinization. The Na^+-independent pH_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^+ gradient, Cl^- gradient or addition of the anion transport inhibitors DIDS (1 mM), SITS (1 mM), α-cyano-4-hydroxycinnamate (4 mM), or probenicid (1 mM). Results suggest that most mouse colonocytes have a previously unreported SCFA transporter which mediates Na^+-independent pH_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).

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In normal mammals, the colonic lumen contains 100–150 mmol/liter total SCFAs (Wrong, Metcalfe-Gibson, Morrison, Ng, and Howard, 1965; Hoverstad, Midtvedt, and Bohmer, 1985; Hoverstad and Midtvedt, 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 pK_as 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 $SCFA^-/HCO_3^-$ 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 (pH_i) homeostasis and insure normal physiologic and metabolic functions of the cell despite constant exposure to SCFAs. The cellular physiology of the colonocyte pH_i 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 pH_i. 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 pH_i 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 pH_i, we observe both Na^+-independent and Na^+-dependent pH_i 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 pH_i alkalinization to promote recovery of pH_i. 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 300 *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 $MgSO_4$, 2 $CaCl_2$, 20 HEPES, 25 mannose, pH 7.4). After dye loading, 50–100 μl aliquots of cells were loaded into a chamber (Montrose, Friedich, 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_i 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_i was used to determine pH_i 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 μm^2 (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_i 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.

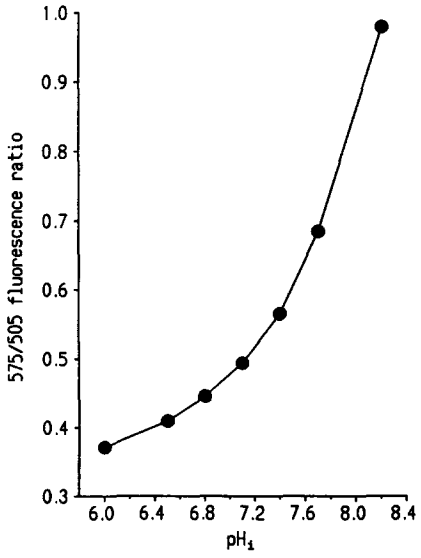

FIGURE 1. Intracellular pH calibration curve of carboxy SNARF-1 loaded colonocytes. Isolated colonocytes were loaded with SNARF-1 and mounted on the stage of the digital imaging microscope as described in Methods. Dual excitation fluorescence ratios (575/505 nm) were calibrated versus pH_i by exposing colonocytes to 10 μM nigericin and 120 mM K^+ solutions at the indicated medium pH values. Data are means of 18 colonocytes from two experiments. All standard errors are smaller than the symbols.

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_i

Similar to other epithelial and nonepithelial cell types (Nakhoul and Boron, 1988; Grinstein, Goetz, Furuya, Rothstein, and Gelfand, 1984; Montrose, Knoblauch, and Murer, 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

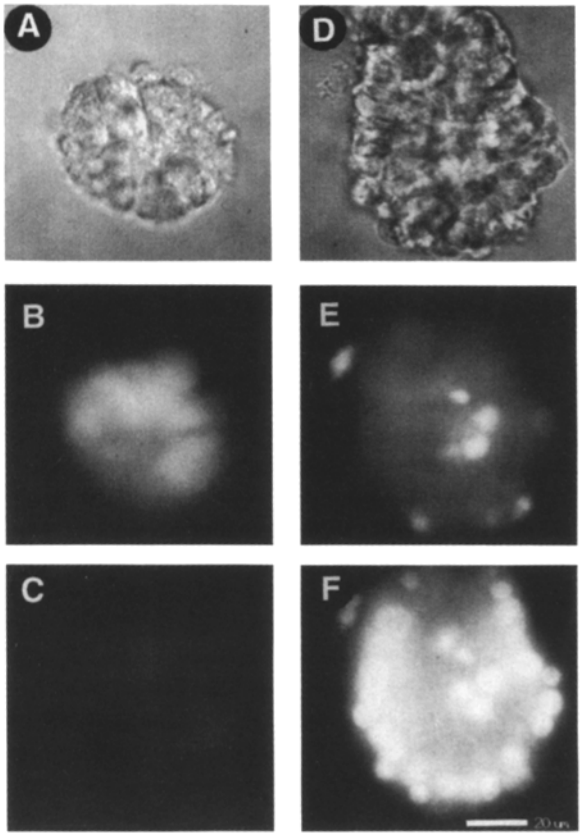

FIGURE 2. SNARF-1 loading and membrane integrity of isolated crypt colonocytes. Results from two crypt fragments loaded with SNARF-1 are presented separately in *A–C* versus *D–F*. (*A–C*) Transmitted light image shows colonocytes in an isolated crypt fragment (*A*). Fluorescence image of SNARF-1 fluorescence signals in the same group of colonocytes under 505 nm excitation (*B*). An image of fluorescence collected under identical conditions of camera gain after 5-min incubation with 10 μM digitonin (*C*). As shown, cytosolic SNARF-1 is released by digitonin, and remaining fluorescence is not punctate. (*D–F*) Transmitted light image shows a second crypt fragment (*D*). In this experiment, cells were superfused with medium containing 5 μM propidium iodide (PI). Fluorescence image shows PI fluorescence (487 nm excitation, 620–670 nm emission) in the same group of colonocytes (*E*). Only a few positive stained nuclei (dead cells) were observed. An image of fluorescence was collected at identical camera gain 10 min after 50 μM digitonin (*F*), and shows many more colonocytes stained positive with propidium iodide. Size bar, 20 μm.

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, pH_i recovery was measured over a range of pH_i 6.7–7.0 and the initial rate of Na^+-independent pH_i recovery was found to be 65 ± 8% (mean ± SEM,

n = 44 cells) of the total pH_i 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 pH_i recovery which was <12% of the total alkalinization rate, suggesting that Na^+-independent pH_i recovery was expressed at a high level in the majority of isolated colonic crypt cells.

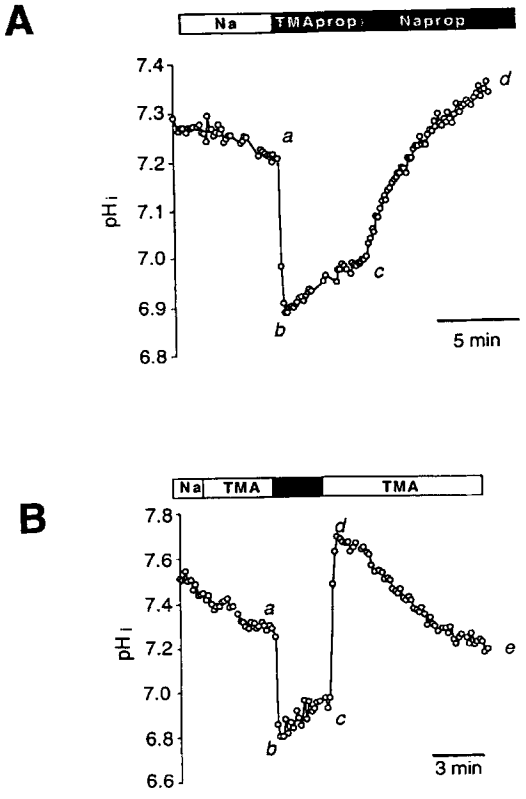

FIGURE 3. Intracellular pH response of colonocytes to propionate. Colonocytes were loaded with SNARF-1, mounted on the microscope and multiple cells studied simultaneously by digital imaging. Dual excitation fluorescence ratios were converted to pH_i using a calibration curve (Fig. 1). (*A*) Na^+-dependent and Na^+-independent pH_i recovery of a single colonocyte exposed to propionate. Cells were first equilibrated in Na medium. When exposed to an isosmotic medium containing 130 mM tetramethylammonium propionate (*TMAprop*), an immediate pH_i acidification (*ab*) was evident. A Na^+-independent pH_i recovery (*bc*) followed the acidification. When Na^+ was introduced into the perfusion with 130 mM sodium propionate (*Naprop*) replacing 130 mM TMAprop, pH_i recovery accelerated (*cd*). (*B*) The pH_i response of colonocytes after the addition and subsequent removal of propionate from Na^+-free perfusates. Addition of 65 mM TMAprop (*black bar*, 65 mM TMAprop, pH 7.4) caused a rapid decrease in pH_i (*ab*) followed by a Na-independent pH_i recovery (*bc*). Removing TMAprop produced a rapid increase in pH_i above the starting pH_i (pH_i overshoot, *cd*), followed by a gradual decrease in pH_i (*de*) back to the basal level (pH 7.2). The data presented are the average of six colonocytes in a single experiment. Similar results were obtained from more than 30 experiments.

As shown qualitatively in Fig. 3 *B*, colonocytes started with an initial pH_i 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 pH_i alkalinization mechanism allowed colonocytes to recover pH from an average acidified pH_i of 6.64 ± 0.03 to a steady state pH_i of 7.01 ± 0.02 (mean ± SEM, n = 55 cells). Upon subsequent removal of propionate from the perfusates, pH_i rapidly alkalinized (Fig. 3 *B*, *cd*) and then slowly returned to the resting pH_i observed

before propionate exposure (Fig. 3 *B, de*). Because the alkalinization raised pH_i to a level higher than starting pH_i in TMA medium, we will use the term 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 Na^+-independent pH_i recovery. In the first experiment, the concentration of nonionized

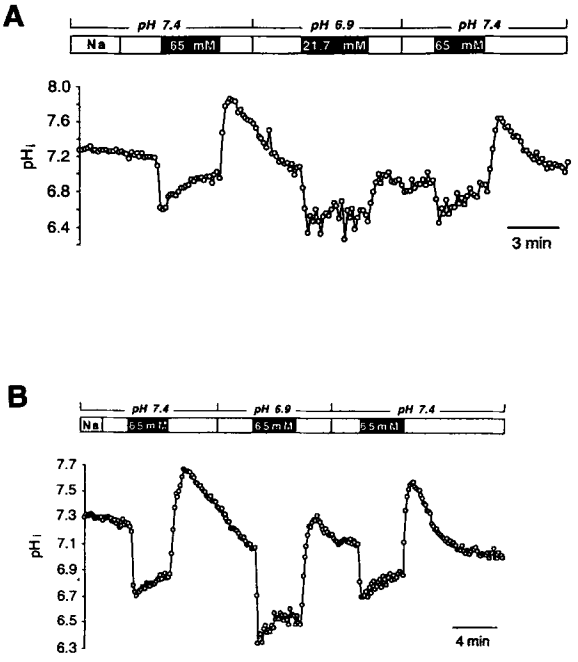

FIGURE 4. Dependence of the pH_i response on the molecular forms of propionate. (*A*) Using the Henderson-Hesselbach equation and the known pK_a of propionate (pK_a = 4.88) two TMAprop solutions (65 mM TMAprop at pH 7.4 and 21.7 mM at pH 6.9) were designed to have the same concentration of nonionized propionate (0.65 mM) but a threefold difference in propionate anion concentration. Acidification was similar after exposure cells to either solution, but the Na^+-independent pH_i recovery and pH_i overshoot were greatly diminished after 21.7 mM propionate exposure. The pH_i trace represents average of nine colonocytes in a single experiment. Identical results were observed from three experiments. (*B*) Acidification was dependent on nonionized SCFA. Two media containing 65 mM propionate were created such that the concentration of nonionized propionate was threefold different (1.95 mM at pH 6.9 vs 0.65 mM at pH 7.4). The difference of anionic propionate between the two solutions was negligible. The acidification of colonocytes in pH 6.9 propionate was more than that in pH 7.4 propionate, but the Na^+-independent pH_i recovery was still observed. The pH_i trace represents average of 17 colonocytes in a single experiment. Similar results were observed from three experiments. (*Open bars*) TMACl medium (pH 7.4); Na: Na medium.

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 pH and total propionate concentration simultaneously according to the Hendeson-Hasselbach equation (pK_a = 4.88). As shown qualitatively in Fig. 4 *A*, the acidification due to 21.7 mM propionate at pH 6.9 (0.48 ± 0.03 pH units, mean ± SEM, n = 20 cells) was not significantly different in a paired comparison with the acidification caused by 65 mM propionate at pH 7.4

(0.54 ± 0.02 pH units). 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. 4 *B*, 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. 4 *B* (because the rate of Na^+-independent pH_i recovery may be pH_i sensitive). Combined with the results of Fig. 4 *A*, 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. 4 *A*). To quantify the magnitude of the overshoot alkalinization, the pH_i overshoot was

defined as the maximal ΔpH_i caused by propionate removal minus the ΔpH_i of initial acidification (in Fig. 3 *B*, overshoot = *cd*−*ab*). When the Na^+-independent pH_i recovery was inhibited by reduction of extracellular propionate anion, the overshoot was also significantly reduced (65 mM/pH 7.4, 0.32 ± 0.03 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 alkalinization of pH_i after propionate removal. Experiments compared estimates of intrinsic buffering capacity generated from acidification upon propionate addition vs alkalinization 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 $\Delta pH < 0.3$). 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 esti-

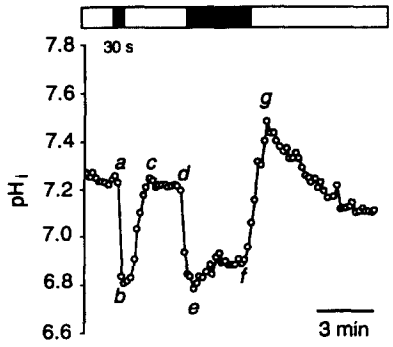

FIGURE 5. Exposure time dependence of propionate activated Na^+-independent pH_i recovery and pH_i overshoot. Cells were perfused either with TMACl medium (*open bars*) or medium containing 65 mM TMAprop (*black bars*) at pH 7.4. Brief (30 s) exposure of colonocytes to 65 mM TMAprop produced acidification (*ab*), but there was no time for the pH_i recovery, and there was no pH_i overshoot upon the replacement of TMAprop by TMACl (*bc*). Longer exposure to propionate showed acidification (*de*) and Na^+-independent pH_i recovery (*ef*) in the presence of 65 mM TMAprop, and the pH_i overshoot (*fg*) upon the removing of propionate. This pH_i trace was an average of six colonocytes in a single experiment. Similar results observed in three experiments.

mated 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 pH_i overshoot is nonionic diffusion (or a protein-mediated flux of nonionized propionate which involves no other titratable acid/base equivalents).

The observed pH_i dependence of buffering capacity was unusual, because most cells have increased buffering capacity at lower pH_i. 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 pH_i-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 pH_i change upon propionate addition would be smaller, and the resultant calculated buffering capacity larger, than the real value. Thus, corruption of measurements by

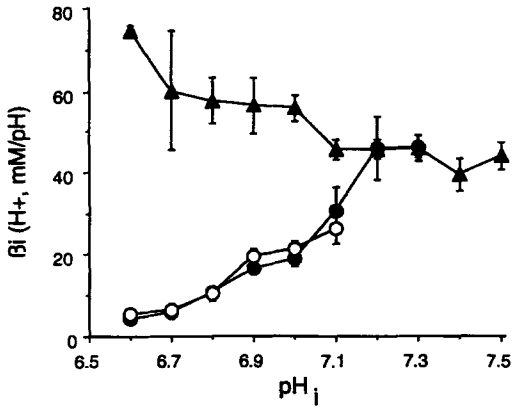

FIGURE 6. Comparison of intrinsic cellular buffering capacity (β_i) calculated from either propionate or ammonium exposure. Colonocytes were exposed to variable concentration of propionate (8–32 mM) to vary the extent of acidification (during propionate addition) and alkalization (during propionate removal). Measurements separately compare results from addition (●) vs removal (○) of propionate from perfusates. Cells were exposed to TMA-Cl medium supplemented with 20 mM NH_4Cl to measure pH_i excursions upon NH_4Cl addition (▲). All values from NH_4Cl addition and propionate addition are collected from the same 65 cells. Intrinsic buffering capacity was calculated by $\beta_i = \Delta[H^+]_i/\Delta pH_i$. $\Delta[H^+]_i$ was estimated by calculating $[prop]_i$ or $[NH_4]_i$ with the Hendeson-Hasselbach equation based on experimental data and known pK_as (propionate = 4.88, ammonium = 9.3) (Roos and Boron, 1981; Watson et al., 1991; Rowe et al., 1994). Propionate removal was quantified in four experiments measuring 38 total colonocytes; propionate addition and NH_4Cl addition were quantified in seven experiments measuring 65 colonocytes. Results are presented as mean ± SEM, n = 6–15 measurements in each pH range.

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.

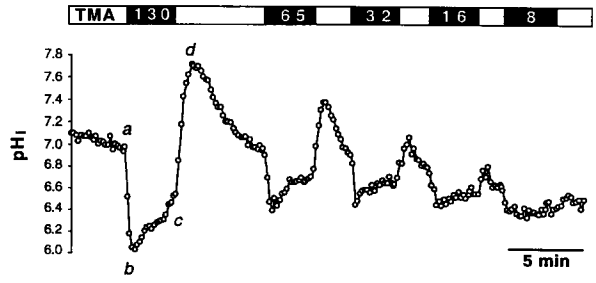

FIGURE 7. Propionate concentration dependency of colonocyte pH_i response. Results are from a single colonocyte exposed successively to 130 to 8 mM TMA-propionate in isosmotic media (TMACl substituted mol:mol by TMAprop). The concentration dependency of acidification (*ab*), Na^+-independent pH_i recovery (*bc*) and pH_i overshoot (*cd*) are shown qualitatively throughout the pH_i trace. (*Open bars*) TMACl medium (pH 7.4); (*black bars*) indicated concentration of TMAprop (pH 7.4).

The acidification was measured as the maximal difference between the pH_i 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 ΔpH of acidification was transformed into a net H^+ increase ($\Delta pH \times \beta_i$, 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 pH_i recovery rate, in response to the same range of

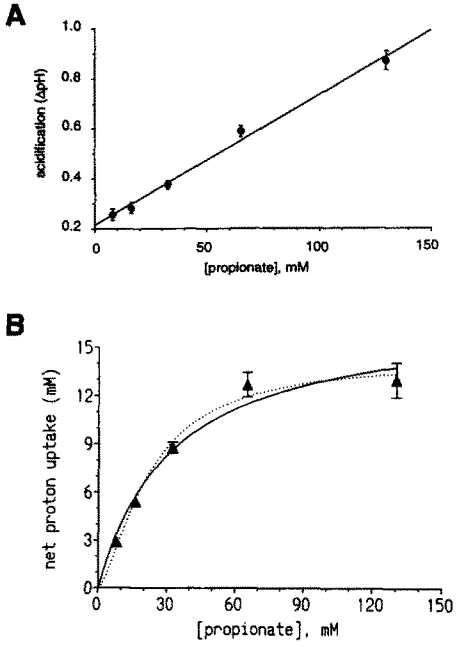

FIGURE 8. Propionate concentration dependency of pH_i acidification. Data from 38 colonocytes in four different experiments were collected as described in Fig. 7. In two experiments, colonocytes were sequentially exposed to descending propionate concentrations (as in Fig. 7), in the other two experiments increasing propionate concentrations were used, and 19 cells were measured from each of the two experiment procedures. (*A*) Data points are the maximum acidification (ΔpH) observed immediately after exposure to the indicated propionate concentration (mean ± SEM, $n = 38$ cells). A linear relationship was evident between the acidification and the concentration of TMAprop. (*B*) Net H^+ influx during the acidification showed a saturable relationship vs propionate. The line is a nonlinear least squares fit to the Michaelis-Menten equation ($K_i = 32$ mM). Net H^+ uptake during acidification was estimated by multiplying ΔpH_i of the acidification by cellular buffering capacity at the same pH_i (values of β_i taken from propionate-deduced values of Fig. 6). Results are mean ± SEM ($n = 38$).

propionate concentrations, was measured as the slope of initial linear phase of the pH_i recovery (pH/min), and converted to a net H^+ efflux rate (mM/min) by multiplication with the total buffering capacity ($\beta_i + \beta_{scfa}$) at the initial pH_i of the measurement.[1] As shown in Fig. 9 *A*, data showed a tendency to saturation at high

[1] β_i at the appropriate pH_i value was determined from propionate data in Fig. 6. β_{prop} was estimated as 2.303* [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 Michaeis-Menten equation, but were well fit by the Hill-equation (K_t = 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_i changes are likely to involve both nonionic and ionic SCFA fluxes (see Discussion), and (*c*) pH_i varies among tested SCFA concentrations (rates of Na^+-independent pH_i recovery may be pH_i-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

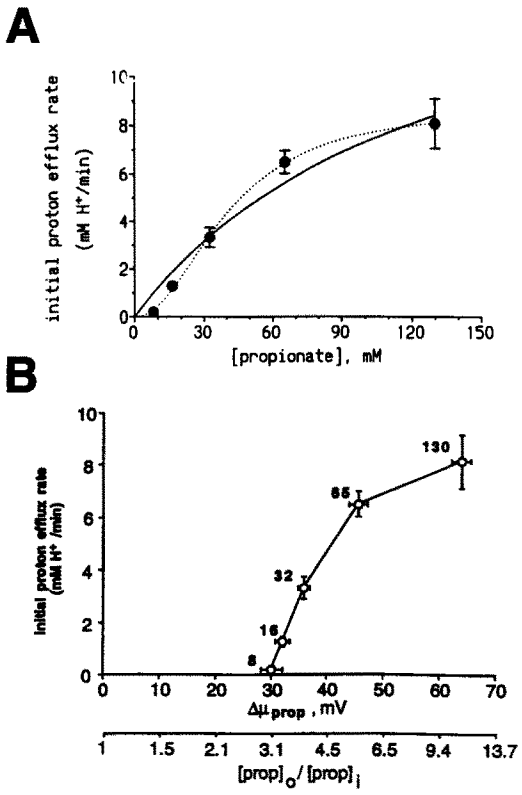

FIGURE 9. Propionate concentration dependency of Na^+-independent pH_i recovery. (*A*) Initial rate of H^+ efflux (measured during the initial 2 min of Na^+-independent pH_i recovery) saturates vs medium propionate concentration. Results were expressed as mean ± SEM (n = 38 cells). Two curve fits are presented in which data are modeled to Michaelis-Menten kinetics (*solid line;* K_t = 132 mM) or the Hill equation (*dotted line;* K_t = 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 ($\Delta\mu$ *prop*) and estimated transmembrane propionate gradient ($[prop]_o/[prop]_i$). 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_i 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_i overshoot was an indirect measure of propionate uptake, we appraised this as an independent measure of the Na^+-independent pH_i recovery. As shown in Fig. 10 *A*, the linear correlation between the magnitude of pH_i overshoot and net H^+ efflux was significant (r = 0.97). To estimate

the H^+ efflux (in millimolar) during a pH_i overshoot, the overshoot ΔpH was multiplied by intrinsic buffer capacity (β_i) 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 pH_i recovery in Fig. 9. The fit to the Hill equation results in a similar K_t (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 pH_i.

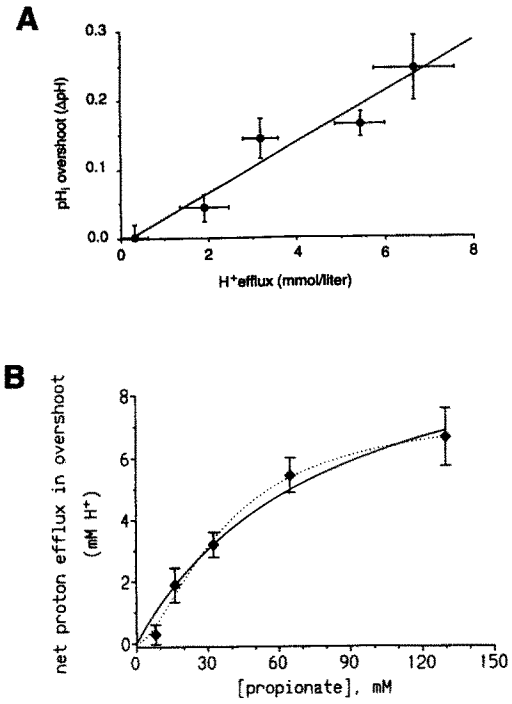

FIGURE 10. Propionate concentration dependency of pH_i overshoot. (*A*) A linear correlation was observed between the magnitude of the pH_i overshoot (ΔpH_i) and the net H^+ efflux. Net H^+ efflux was calculated from measurements of cellular buffering capacity multiplied by the observed ΔpH_i overshoot (mean ± SEM, n = 38 cells). (*B*) A saturable relationship was observed between the net H^+ efflux during pH_i overshoot and concentration of propionate. Two curve fits are presented in which data are modeled to Michaelis-Menten kinetics (*solid line;* K_t = 80 mM) or the Hill equation (*dotted line;* K_t = 37 mM, Hill coefficient = 1.6) (mean ± SEM, n = 38 cells).

Potential Substrates and Inhibitors of Na^+-independent pH_i Recovery

To further test whether SCFAs directly mediate Na^+-independent pH_i 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 pH_i of colonocytes (Fig. 11). When exposed to these SCFAs, colonocytes acidified promptly, and then a Na^+-independent pH_i recovery was observed to alkalinize pH_i to ~pH 7.0. Upon SCFA removal, immediate alkalinization and a pH_i 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_i changes are due to transport, not metabolism, of the SCFAs.

Several weak acids did not elicit Na^+-independent pH_i recovery. As shown in Fig. 11, *A* and *C*, the weak acids formate and lactate (2-hydroxy propionate) caused

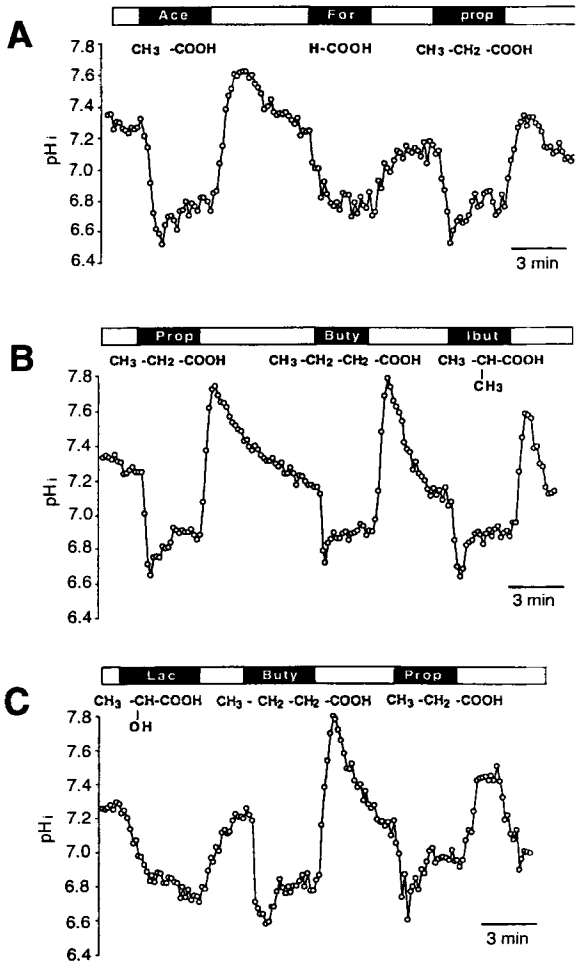

FIGURE 11. Substrate specificity of the SCFA transporter responsible for Na^+-independent pH_i recovery. All SCFAs and naturally occurring weak acids were tested at 65 mM and pH 7.4. Propionate was used as the internal control in all experiments to allow comparison between different preparations. All experiments were repeated three times and identical results obtained. Typical results from single experiments are presented. (*A*) Acetate (*Ace*) acidified colonocytes and activated Na^+-independent pH_i recovery and pH_i overshoot. Formate (*For*) acidified colonocytes but neither activated Na^+-independent pH_i recovery nor elicited pH_i overshoot. The pH_i trace represents average of five colonocytes. (*B*) Both *n*-butyrate (*Buty*) and *iso*-butyrate (*Ibut*) acidified colonocytes, and activated Na^+-independent pH_i recovery and pH_i overshoot similar to propionate. The pH_i trace presents average of four colonocytes. (*C*) Lactate (*Lac*) acidified colonocytes, but did not activate Na^+-independent pH_i recovery or the pH_i overshoot. The pH_i trace represents average of eight colonocytes. (*Open bars*) TMACl medium (pH 7.4).

intracellular acidification, but Na^+-independent pH_i 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 pH_i recovery. This demonstrates

that the Na^+-independent pH_i 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. 12 *A*, this compound acidified colonocytes as effectively as propionate (113 ± 4% of propionate response, $n = 18$ cells), but Na^+-independent pH_i recovery and pH_i 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

TABLE I

*Effect of Different Monocarboxylic Acids on pH_i of Mouse Colonocytes**

	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 ± 0.0	(5)

*SNARF-1 loaded colonocytes were exposed to 65 mM of the indicated weak acid as a TMA salt, and the subsequent changes in pH_i 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 pH_i 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 ($P > 0.05$).

similar apparent decrease in Na^+-independent pH_i 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 pH_i (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 pH_i (data not shown).

To examine sensitivity of the SCFA transporter to known transport inhibitors, several drugs were tested for their effect on Na^+-independent pH_i 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),

SCFA-stimulated volume regulation (Rowe, Blackmon, and Montrose, 1993), and some $SCFA^-/HCO_3^-$ 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

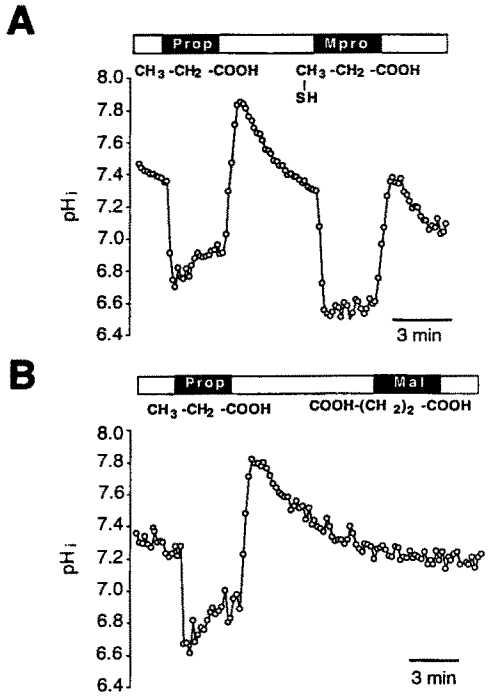

FIGURE 12. Effect of nonphysiologic weak acids on intracellular pH of colonocytes. (*A*) 3-mercaptopropionate (65 mM) acidified colonocytes but did not effectively activate Na^+-independent pH_i recovery or cause pH_i overshoot when removed from the perfusate. Average results from six colonocytes in 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*) TMACl medium (pH 7.4).

Na^+-independent pH_i recovery was calculated from the initial 2 min of the pH_i recovery and expressed as ΔpH/min. The effects of inhibitors were estimated as percent of control pH_i 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 recovery rate. In addition (data not shown), no drug affected base line pH_i before propionate exposure, or affected the acidification caused by propionate. Results suggest that the Na^+-independent pH_i recovery of mouse colonocytes is unlikely to be mediated by previously described SCFA transporters.

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. 14 *A* 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. 14 *B*, 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

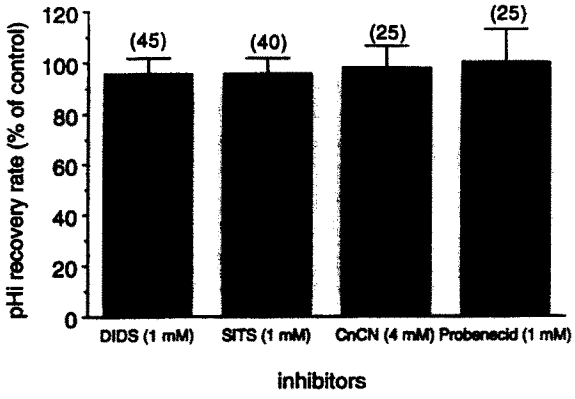

FIGURE 13. Effects of known anion transport inhibitors on the SCFA transporter. Initial rates of Na^+-independent pH_i recovery of the same colonocytes exposed to 65 mM TMAprop (*control*) vs 65 mM TMAprop plus a specific inhibitor (concentration marked in the figure). Rates were determined from initial 2 min of the pH_i recovery in each condition. To insure inhibitors reached colonocytes, cells were always exposed to inhibitors (in TMACl) for 2–3 min before exposing cells to TMAprop plus inhibitor. Effects of inhibitors were expressed as percentage of the control (pH_i recovery rate in 65 mM TMAprop alone). Numbers on the top of each column indicate number of cells from which data were collected. No significant inhibition of the SCFA transporter by these inhibitors was observed.

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

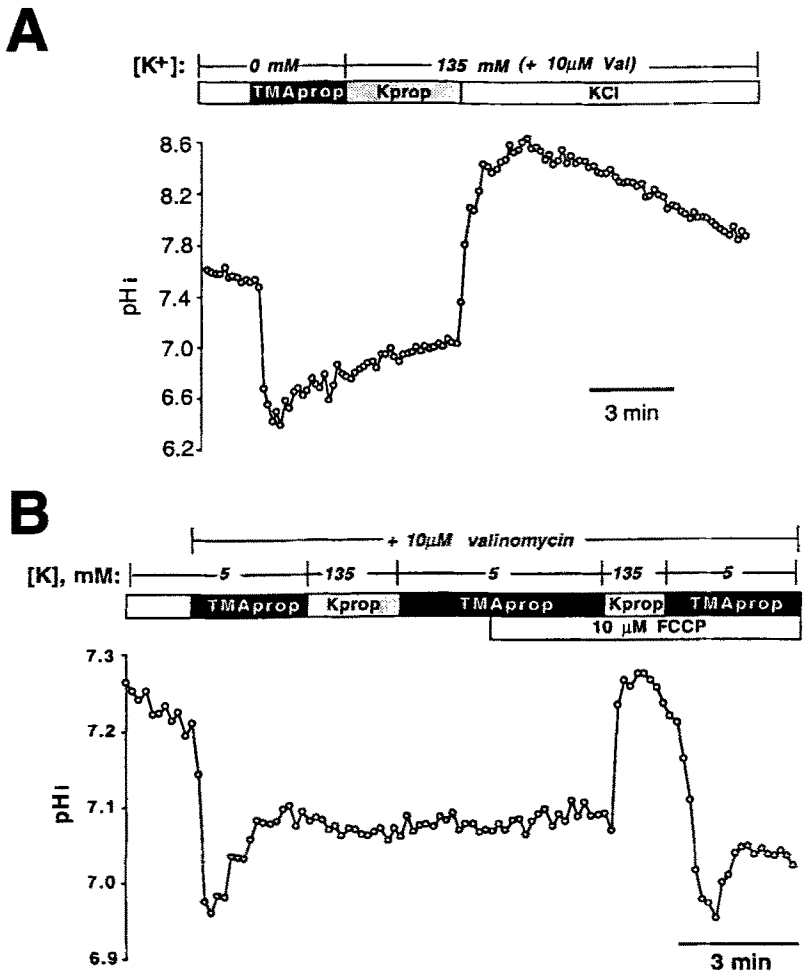

FIGURE 14. Effect of medium K^+ and membrane potential on the SCFA transporter. (*A*) Colonocytes were preincubated in K^+-free medium for 30 min before experiments and initially perfused in K^+-free TMACl medium (*open bar*). Adding 135 mM TMAprop in a K^+-free medium (*black bar*) produced acidification and Na^+-independent pH_i 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 pH_i recovery. The pH_i 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*, pH_i 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*).

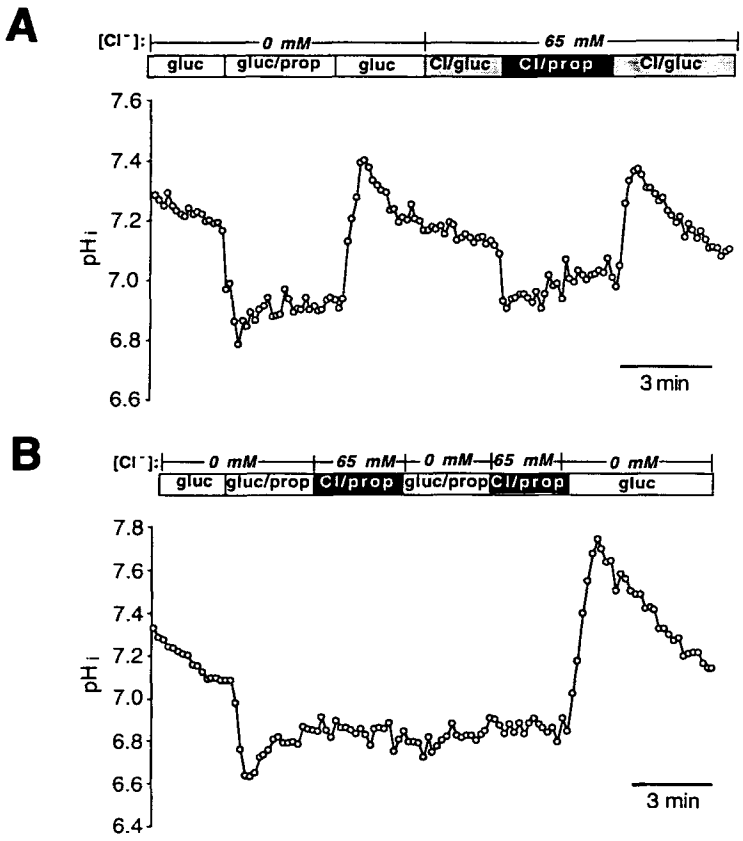

FIGURE 15. Effect of changes in transmembrane Cl^- gradient on the SCFA transporter. Colonocytes were preincubated in Cl^--free medium (gluc = Cl^- replaced mol:mol with gluconate) for 30 min before experiments and initially perfused in the same medium. (*A*) Cells were transiently exposed to a Cl^--free 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 pH_i recovery or pH_i 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 pH_i trace presents average response of 14 colonocytes in a single experiment. (*B*) During the steady state pH_i after Na^+-independent pH_i recovery, switching perfusion between Cl^--free (*gluc/prop*) and Cl^--containing (*Cl/prop*) TMAprop did not affect steady state. The pH_i 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 pH_i 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 pH_i 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*) nonionic 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 pH_i 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 NH_4 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 pH_i recovery). Experimental evidence suggests this choice was correct. Similar propionate activation kinetics were estimated from initial proton efflux rates (Fig. 9 *A*) vs proton efflux estimated from the overshoot (Fig. 10 *B*); even though these two data sets use buffering capacity values from an acidic or alkaline pH_i range, respectively. This internal consistency is unlikely to be observed if the pH_i 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 pH_i 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, Helmle-Kolb, Murer, and Scharrer, 1993; 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 pH_i recovery could be detected either

as a pH_i recovery during SCFA exposure, or as an alkaline overshoot of pH_i 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_i 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_i overshoot was a (direct or indirect) consequence of Na^+-independent pH_i recovery. Overshoots were not observed (or were greatly diminished) if Na^+-independent pH_i recovery was limited by (*a*) brief pulses of propionate (Fig. 5); (*b*) coincident lowering of propionate anion concentration and medium pH (Fig. 4*A*); or (*c*) exposure to weak acids which did not demonstrate Na^+-independent pH_i recovery (e.g., lactate, 3-mercaptopropionate or formate). Further, estimates of the propionate activation kinetics were similar in measurements of pH_i recovery rates (Fig. 9) or the magnitude of the associated overshoot (Fig. 10). These results suggest that pH_i overshoots are valid to quantify Na^+-independent pH_i 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_i recovery was mediated by a SCFA transport process in the plasma membrane, and not a SCFA-independent pH_i alkalinization mechanism stimulated by cellular acidification. First, the Na^+-independent pH_i recovery demonstrated saturation by extracellular SCFA, independent of driving force for net SCFA uptake (Figs. 9 and 10). Most importantly, Na^+-independent pH_i 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_i recovery, and (*b*) suggest that in no case was acidification masking rapid Na^+-dependent pH_i 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_i recovery mechanism. These results are difficult to reconcile with a model in which SCFA anions are impermeant, and Na^+-independent pH_i recovery is mediated by a SCFA-independent mechanism. Results suggest that a saturable, substrate-specific SCFA transporter mediates the Na^+-independent pH_i 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 alkalization 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 alkalization 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 electroneutral, 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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REFERENCES

Aronson, P. S., J. Nee, and M. A. Suhm. 1982. Modifier role of internal H^+ in activating the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature.* 299:161–163.

Bassnett, S., L. Reinisch, and D. C. Beebe. 1990. Intracellular pH measurement using single excitation-dual emission fluorescence ratios. *American Journal of Physiology.* 258:C171–178.

Bergman, E. N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Reviews.* 70:567–590.

Binder, H. J., and P. Metha. 1989. Short-chain fatty acid stimulate active sodium and chloride absorption in vitro in the rat distal colon. *Gastroenterology.* 96:989–996.

Buckler, K. J., and R. D. Vaughan-Jones. 1990. Application of a new pH-sensitive fluoroprobe (carboxy-SNARF-1) for intracellular pH measurement in small, isolated cells. *European Journal of Physiology.* 417:234–239.

Bugaut, M. 1987. Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. *Comparative Biochemistry and Physiology.* 86B:439–472.

Cummings, J. H., E. W. Pomare, W. J. Branch, C. P. E. Naylor, and G. T. Macfarlane. 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut.* 28:1221–1227.

Diener, M., C. Helmle-Kolb, H. Murer, and E. Scharrer. 1993. Effect of short-chain fatty acids on cell volume and intracellular pH in rat distal colon. *European Journal of Physiology.* 424:216–223.

Engelhardt, W. V., and G. Rechkemmer. 1985. Absorption of inorganic ions and short-chain fatty acids in the colon of mammals. *In* Intestinal Transport: Fundamental and Comparative Aspects. M. Gilles-Baillen, and R. Gilles, editors. Springer-Verlag, New York. 27–45.

Engelhardt, W. V., and G. Rechkemmer. 1992. Segmental differences of short-chain fatty acid transport across guinea-pig large intestine. *Experimental Physiology.* 77:491–499.

Engelhardt, W. V., M. Burmester, K. Hansen, G. Becker, and G. Rechkemmer. 1993. Effects of amiloride and ouabain on short-chain fatty acid transport in guinea-pig large intestine. *Journal of Physiology* 460:455–466.

Feldman, G. M., F. N. Ziyadeh, J. W. Mills, G. W. Booz, and A. Kleinzeller. 1989. Propionate induces cell swelling and K+ accumulation in shark rectal gland. *American Journal of Physiology.* 257:C377–C384.

Gabel, G., S. Vogler, and H. Martens. 1991. Short-chain fatty acids and CO_2 as regulators of Na^+ and Cl^- absorption in isolated sheep rumen mucosa. *Journal of Comparative Physiology B.* 161:419–426.

Garcia, C. K., J. L. Goldstein, R. K. Pathak, R. G. W. Anderson, and M. S. Brown. 1994. Molecular characterization of a membrane transporter for lactate, pyruvate, and other monocarboxylates: implications for the Cori cycle. *Cell.* 76:865–873.

Grinstein, S., J. D. Goetz, W. Furuya, A. Rothstein, and E. W. Gelfand. 1984. Amiloride-sensitive Na^+-H^+ exchange in platelets and leukocytes: detection by electronic cell sizing. *American Journal of Physiology.* 27:C293–C298.

Guggino, W. B., and S. E. Guggino. 1989. Renal anion transport. *Kidney International.* 36:385–391.

Harig, J. M., K. H. Soergel, J. A. Barry, and K. Ramaswamy. 1991. Transport of propionate by human ileal brush-border membrane vesicles. *American Journal of Physiology.* 260:G776–G782.

Holtug, K. 1989. Mechanisms of absorption of short chain fatty acids—coupling to intracellular pH regulation. *Acta Veterinaria Scandinavia.* 86:126–133.

Holtug, K., H. S. Rasmussen, and P. B. Mortensen. 1992. An *in vitro* study of short-chain fatty acid concentrations, production and absorption in pig (*Sus scrofa*) colon. *Comparative Biochemistry and Physiology A. Comparative Physiology.* 103:189–197.

Holtug, K. 1993. Specificity and inhibition of the short-chain fatty acid carrier in colon. *Gastroenterology.* 104:A253. (Abstr.).

Hoverstad, T., T. Midtvedt, and T. Bohmer. 1985. Short-chain fatty acids in intestinal content of germfree mice monocontaminated with *Escherichia coli* of Clostridium difficile. *Scandinavia Journal of Gastroenterology.* 20:373–380.

Hoverstad, T., and T. Midtvedt. 1986. Short-chain fatty acids in germfree mice and rats. *Journal of Nutrition.* 116:1772–1776.

Jackson, M. J., A. M. Williamson, W. A. Dombrowski, and D. E. Garner. 1978. Intestinal transport of weak electrolytes: determinants of influx at the luminal surface. *Journal of General Physiology.* 71:301–327.

Kaunitz, J. D. 1988. Preparation and characterization of viable epithelial cells from rabbit distal colon. *American Journal of Physiology.* 254:G502–G512.

Macfarlane, G. T., and J. H. Cummings. 1991. The colonic flora, fermentation, and large bowel digestive function. *In* The Large Intestine: Physiology, Pathophysiology, and Disease. S. F. Philips, J. H. Pemberton, and R. G. Shorter, editors. Raven Press, New York. 51–92.

Macfarlane, G. T., G. R. Gibson, and J. H. Cummings. 1992. Comparison of fermentation reactions in different regions of the human colon. *Journal of Applied Bacteriology.* 72:57–64.

Mascolo, N., V. M. Rajendran, and H. J. Binder. 1991. Mechanism of short-chain fatty acid uptake by apical membrane vesicles of rat distal colon. *Gastroenterology.* 101:331–338.

Montrose, M. H., and G. A. Kimmich. 1986. Quantitative use of weak bases for estimation of cellular pH gradients. *American Journal of Physiology* 247:C293–C298.

Montrose, M. H., T. Friedrich, and H. Murer. 1987. Measurements of intracellular pH in single LLC-PK$_1$ cells: recovery from an acid load via basolateral Na^+/H^+ exchange. *Journal of Membrane Biology.* 97:63–78.

Montrose, M. H., C. Knoblauch, and H. Murer. 1988. Separate control of regulatory volume increase and N^+/H^+ exchange by cultured renal cells. *American Journal of Physiology.* 25:C76–C85.

Montrose, M. H., M. A. Condrau, and H. Murer. 1989. Flow cytometric analysis of intracellular pH in cultured opossum kidney (OK) cells. *Journal of Membrane Biology.* 108:31–43.

Nakhoul, N. L., A. G. Lopes, J. R. Chaillet, and W. F. Boron. 1988. Intracellular pH regulation in the S3 segment of the rabbit proximal tubule in HCO_3^--free solutions. *Journal of General Physiology.* 92:369–393.

Nakhoul, N. L., and W. F. Boron. 1988. Acetate transport in the S3 segment of the rabbit proximal tubule and its effect on intracellular pH. *Journal of General Physiology.* 92:395–412.

Naupert, C., and K. Rommel. 1975. Absorption of short and medium chain fatty acids in jejunum of the rat. *Zeitschrift für Klinische Chemie und Klinische Biochemie.* 13:553–562.

Petersen, K. U., J. R. Wood, G. Schulze, and K. Heintze. 1981. Stimulation of gallbladder fluid and electrolyte absorption by butyrate. *Journal of Membrane Biology.* 62:183–193.

Poole, R. C., and A. P. Halestrap. 1993. Transport of lactate and other monocarboxylates across mammalian plasma membranes. *American Journal of Physiology.* 264:C761–782.

Rajendran, V. M., and H. J. Binder. 1994. Apical membrane Cl-butyrate exchange: mechanism of SCFA stimulation of active Cl absorption in rat distal colon. *Journal of Membrane Biology.* 141:51–58.

Reynolds, D. A., V. M. Rajendran, and H. J. Binder. 1993. Butyrate-stimulated [^{14}C]-butyrate uptake in basolateral membrane vesicles of rat distal colon. *Gastroenterology.* 105:725–732.

Ronnau, K., D. Guth, and W. V. Engelhardt. 1989. Absorption of dissociated and undissociated short-chain fatty acids across the colonic epithelium of guinea-pig. *Quarterly Journal of Experimental Physiology.* 74:511–519.

Roos, A., and W. F. Boron. 1981. Intracellular pH. *Physiological Reviews.* 61:296–434.

Rosenberg, S. O., T. Fadil, and V. L. Schuster. 1993. A basolateral lactate/H^+ co-transporter in Madin-Darby Canine Kidney (MCDK) cells. *Biochemical Journal.* 289:263–268.

Rowe, W. A., D. L. Blackmon, and M. H. Montrose. 1993. Propionate activates multiple ion transport mechanisms in the HT29-18-Cl human colon cell line. *American Journal of Physiology.* 265:G564–G571.

Rowe, W. A., M. J. Lesho, and M. H. Montrose. 1994. Polarized Na^+/H^+ exchange function is pliable in response to transepithelial gradients of propionate. *Proceedings of the National Academy of Sciences, USA.* 91:6166–6170.

Schmitt, M. G., K. H. Soergel, and C. M. Wood. 1976. Absorption of short chain fatty acids from the human jejunum. *Gastroenterology.* 70:211–215.

Sellin, J. H., and R. DeSoignie. 1990. Short-chain fatty acid absorption in rabbit colon *in vitro*. *Gastroenterology.* 99:676–683.

Siebens, A. W., and W. F. Boron. 1987. Effect of electroneutral luminal and basolateral lactate transport on intracellular pH in salamander proximal tubules. *Journal of General Physiology.* 90:799–831.

Stein, J., O. Schröder, V. Milovic, and W. F. Caspary. 1995. Mercaptopropionate inhibits butyrate uptake in isolated apical membrane vesicles of the rat distal colon. *Gastroenterology.* 108:673–679.

Thomas, J. A., R. N. Bushbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 18:2230–2238.

Walter, A., and J. Gutknecht. 1984. Monocarboxylic acid permeation through lipid bilayer membrane. *Journal of Membrane Biology.* 77:255–264.

Watson, A. J. M., S. Levine, M. Donowitz, and M. H. Montrose. 1991. Kinetics and regulation of a polarized Na^+-H^+ exchanger from Caco-2 cells, a human intestinal cell line. *American Journal of Physiology.* 261:G229–G238.

Weaver, G. A., J. A. Krause, T. L. Miller, and M. J. Wolin. 1989. Constancy of glucose and starch fermentation by two different human faecal microbial communities. *Gut.* 30:19–25.

Weigand, E, J. W. Young, and A. D. McGilliard. 1975. Volatile fatty acid metabolism by rumen mucosa from cattle fed hay or grain. *Journal of Dairy Science.* 58:1294–1300.

Wrong, O. M., A. Metcalfe-Gibson, R. B. I. Morrison, S. T. Ng, and A. V. Howard. 1965. *In vivo* dialysis of faeces as a method of stool analysis. I. Technique and results in normal subjects. *Clinical Science.* 28:357–375.