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Role of monocarboxylic acid transport in intracellular pH regulation of isolated proximal cells

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Isolated proximal cells were prepared from rabbit kidney cortex by mechanical dissociation. The intracytoplasmic pH (pH_i) was measured in HCO_3^--free media (external pH (pH_e), 7.3) using the fluorescent dye 2,7-biscarboxyethyl-5,6-carboxyfluorescein (BCECF). Cells were acid-loaded by the nigericin technique. Addition of 70 mM Na^+ to the cells caused a rapid pH_i recovery, which was blocked by 0.5 mM amiloride. When the cells were exposed to 5 mM sodium butyrate in the presence of 1 mM amiloride, the H^+ efflux was significantly increased and followed Michaelis-Menten kinetics. Increasing pH_e from 6.4 to 7.6 at a constant pH_i of 6.4 enhanced the butyrate activation of the H^+ efflux. Increasing pH_i from 6.5 to 7.2 at a constant pH_e of 7.2 reduced the butyrate effect. ^{22}Na uptake experiments in the presence of 1 mM amiloride showed that 1.5 mM butyrate increased the Na^+ flux in the proximal cells (pH_i 7.10). The efficiency of monocarboxylic anions in promoting a pH_i recovery increased with the length of their straight chain (acetate < propionate < butyrate < valerate). The data show that when the Na^+/H^+ antiporter is blocked, the proximal cells can regulate their pH_i by a Na^+-coupled absorption of butyrate followed by non-ionic diffusion of butyric acid out of the cell and probably also by OH^- influx by means of the OH^-/anion exchanger.

Introduction

The sensitivity of many cellular events to pH variations necessitates efficient regulation of the intracellular pH. Like most of the mammalian cells, epithelial cells possess an Na^+/H^+ antiport undertaking an important part of the proton extrusion from the cells. The characteristics of this exchanger along the proximal tubule have been extensively studied both in vivo [1,2] and in vitro [3–6]. Other mechanisms modulating active H^+ secretion across the luminal membrane of the proximal cells have also been identified [7–12]. One of these was shown by Blomstedt and Aronson [8] to be an organic anion/hydroxyl ion antiporter, present in brush borders isolated from the rat kidney. This antiporter could operate simultaneously with the Na^+-dependent transporter of organic anions [13] in the luminal membrane, and could effect net acidification of proximal tubular fluid. In addition, Na^+-coupled adsorption of organic anions followed by non-ionic diffusion of the undissociated organic acid back into the lumen could participate in the acidification phenomenon. The aim of the present work was to

Abbreviations: BCECF, 2,7-biscarboxyethyl-5,6-carboxyfluorescein; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; TMA^+, tetramethylammonium; pH_i, intracellular pH; pH_e, external pH.

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investigate the role of such mechanisms in pH regulation in the proximal tubule. For this purpose, we used a viable preparation of proximal cells isolated from rabbit kidney cortex by simple mechanical dissociation [14]. The effects of short-chain monocarboxylic fatty acids (mainly butyrate) on the pH_i recovery of acidified cells were studied by using the fluorescent dye, BCECF.

Materials and Methods

Animals

Adult female New-Zealand white rabbits (body wt., 2.0–2.5 kg) were used for the experiments. All animals were fed a standard diet and had free access to tap water. For cell preparation, kidneys were removed from animals killed by 5 ml pentobarbital and 2500 U heparin (Roussel, France) injected through the vein of the ear.

Isolated proximal cell preparation

Experiments were performed on isolated proximal cells from the kidneys prepared as previously described in our laboratory by Poujeol and Vandewalle [14]. For each experiment, four kidneys were perfused with a RPMI medium (Gibco, U.S.A.) devoid of sodium bicarbonate and buffered with 25 mM Hepes at pH 7.4. Slices from the superficial cortex were then passed through a tissue press and successively filtered through 100, 40 and 20 μm nylon meshes. At the end of the procedure, $(200–400) \cdot 10^6$ cells were obtained. Viability of the cells was assessed by eosin dye exclusion. Cells were prepared for experimentation by centrifuging twice at $150 \times g$ for 5 min in 50 ml RPMI medium, and the final pellet was resuspended in RPMI medium to a concentration of $50 \cdot 10^6$ cells per ml.

The total number of cells was estimated by microscopic observation on 20 μl of cell suspension diluted in 0.5% eosin solution; protein content was measured according to the method of Bradford (Bio-Rad protein assay, Bio-Rad Laboratories, München, F.R.G.) using bovine serum albumin as reference.

Intracellular pH measurements

pH_i determinations with BCECF. The pH_i was measured fluorimetrically using BCECF-loaded cells as previously described [15,16]. The technique has been described in detail [17]. Cells $(50 \cdot 10^6$/ml) were loaded by incubation with 4 μg/ml BCECF (1 μM/ml dimethyl sulfoxide) at 37 °C. After 30 min incubation, they were centrifuged at $150 \times g$ for 5 min and resuspended in fresh RPMI medium before use. Preliminary experiments on the variation of the cellular fluorscence intensity as a function of time indicated that dye leakage did not exceed 7–12%/h at room temperature. This was considered to be negligible, since experiments did not exceed 10 min. Furthermore, washings of the cells were always performed just before the fluorimetric measurements. Fluorescence of the labeled cells was determined in a Perkin-Elmer LS5 fluorescence spectrophotometer at 500 nm for excitation and 530 nm for emission and recorded by a Perkin-Elmer recorder. The pH_i measurements were always made at room temperature in 3 ml quartz cuvettes containing $5 \cdot 10^6$ proximal cells suspended in 2 ml of the appropriate buffer. During measurements, the cells were stirred continuously by means of a magnetic stirrer.

Fluorescence signals relating to intracellular pH changes were calibrated using the K^+/H^+-exchanging ionophore, nigericin [16,18].

Adjustment of pH_i, determinations of the buffering power and of the rate of proton efflux. When necessary, intracellular pH was lowered by suspension of the cells in choline medium (140 mM choline chloride/5 mM KCl/1 mM $CaCl_2$/0.4 mM $MgSO_4$/2 mM glutamine/10 mM glucose/20 mM Hepes (pH 7.4)) with the addition of 0.62 μg/ml of nigericin. Once the desired pH_i value (6.3–6.7) was obtained, acid loading was stopped by the addition of 50 μl bovine serum albumin (200 mg/ml) [18]. The cell-buffering power was determined by the NH_4^+ technique [18,19] and the buffering capacity was calculated according to Roos and Boron [19]. From the above two parameters, (pH_i and buffering power) the proton efflux as a function of time can be calculated as follows: H^+ efflux = buffering power $\times$ pH_i in mmol $H^+ \cdot l^{-1} \cdot min^{-1}$. In all experimental conditions, the fluorescence was recorded continuously and the pH_i measured using the first 30 s in order to calculate the initial rate of proton efflux.

Butyrate and the other monocarboxylic acids tested, were used in the sodium-salt form at a

concentration of 5 mM. These substrates were added to cell suspensions just after the acid loading procedure and 30 s–1 min before addition of NaCl.

Experimental protocol. After the BCECF-loading procedure, the cells were twice rinsed with RPMI solution and again carefully adjusted to $50 \cdot 10^6$/ml. 8–10 aliquots (100 μl) were then acid loaded as described above. An initial calibration was performed and one experimental run was undertaken. At the end of the run, a second calibration was made. The time lapse between dye loading and the second calibration did not exceed 50 min. The operation was repeated at least twice on the same cell preparation. According to the total number of cells available, one or two additional runs were performed. The number of cells in the 100 μl aliquots was controlled several times during the experiments.

^{22}Na uptake measurements

Measurements of ^{22}Na uptake by the isolated cells were made at 37° C by the centrifugation method previously described [14]. After preparation, isolated cells were rinsed three times in a mannitol buffer (280 mM mannitol/5 mM KCl/1 mM $CaCl_2$/0.4 mM $MgSO_4$/2 mM glutamine/20 mM Hepes (pH 7.4)) and kept for 1 h at 37° C in order to ensure cellular Na^+ depletion. Aliquots of 100 μl of cell suspension ($5 \cdot 10^6$ cells) were equilibrated at 37° C and uptake was initiated with the addition of 100 μl mannitol buffer containing 3, 10, 20 and 40 mM ^{22}Na (Amersham International, U.K.) in the absence or the presence of 1.5 mM butyrate. The uptakes were terminated by removing 180 μl aliquots from the suspension and diluting them in 1 ml of ice-cold mannitol stop solution. The cells were centrifuged for 30 s in a Beckman microfuge and the pellet was washed twice. The pelleted cells were dissolved in 500 μl 1 M NaOH and the radioactivity determined by scintillation counting (Intertechnique SL 4000). All incubations were carried out in triplicate. The extracellular water space was measured in each experiment by using [^{3}H]sorbitol, and the sodium data were corrected for this factor. The extracellular volume in a pellet represented between 20 and 30% of the total water volume, determined from the distribution of tritiated water.

Statistical analysis

Values reported in the text are means ± S.E. Student t-test was used for statistical analysis.

Results

Effect of butyrate on sodium-dependent pH_i recovery after acute intracellular acid loading

A typical experiment in which Na^+-induced alkalinization was determined in the presence or

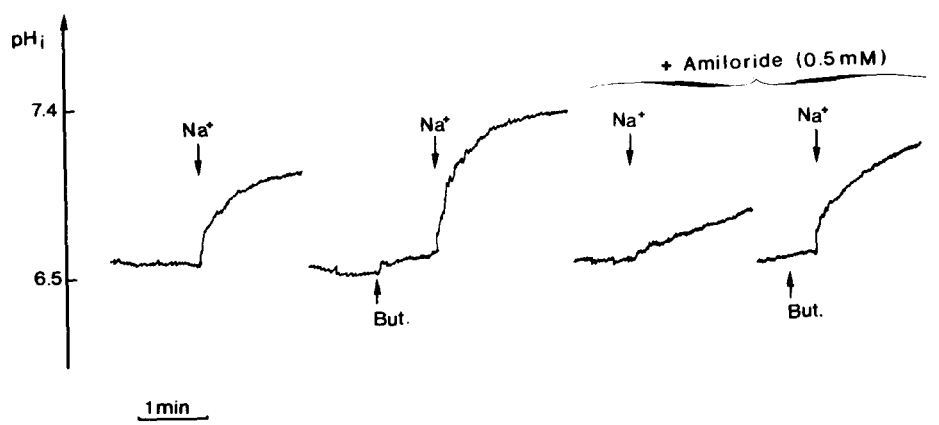


Fig. 1. Effect of butyrate on Na^+-dependent proton efflux; typical fluorescence recordings. Isolated cells loaded with BCECF were resuspended in choline medium ($5 \cdot 10^6$ cells/2 ml) after acidification by the nigericin technique to $pH_i \approx 6.5$. Realkalinization of the cell was induced by addition of sodium chloride (74 mM Na^+) without or with 0.5 mM amiloride, in the presence or the absence of sodium butyrate (to 5 mM). Recordings were made at constant pH_e (7.30) at room temperature.

the absence of butyrate is illustrated in Fig. 1. The BCECF-loaded cells were acidified to pH 6.5 as described in Materials and Methods. As expected, 74 mM Na^+ in the external medium induced a rapid increase of intracellular cell fluorescence because of alkalinization of the cells due to Na^+/H^+ antiporter activation. Addition of 5 mM of butyrate alone (as sodium salt) did not significantly modify the basal fluorescence, but amplified the response to external sodium. When the cells were resuspended in 0.5 mM amiloride solution, the Na^+-induced alkalinization was strongly blocked, but in the presence of butyrate the addition of Na^+ again produced a rapid alkalinization. It appears, therefore, that the action of butyrate on Na^+-dependent pH_i recovery is independent of the amiloride-sensitive Na^+/H^+ antiporter.

To test the possibility that the intracellular alkalinization observed with butyrate is due to metabolic activation, we performed experiments with BCECF-loaded cells pretreated with KCN. The results are illustrated in Fig. 2. Na^+-induced alkalinization was studied in the presence of valerate or butyrate in 1 mM amiloride solutions. Poisoning the cells by 30 min treatment with 0.5 mM KCN was without any significant effect on valerate- or butyrate-induced Na^+-dependent alkalinization.

Experiments were then carried out to determine the cation selectivity of the phenomenon. The rate of change of pH_i upon addition of 74 mM of the chloride salt of the test cation was measured in cells acidified by the nigericin technique with or without butyrate stimulation. In all cases the incubation solution contained 1 mM amiloride. The

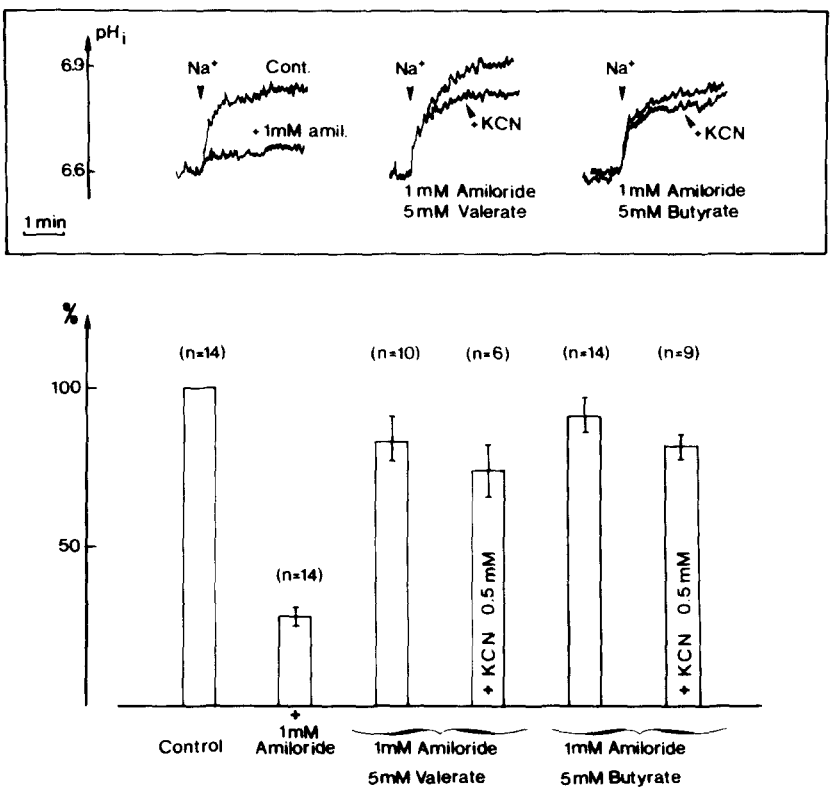


Fig. 2. Effect of potassium cyanide on the Na^+-dependent proton efflux induced by butyrate. Inset: typical fluorescence traces of BCECF acid-loaded cells; effect of potassium cyanide. Isolated cells were acidified by the nigericin technique to $pH_i \approx 6.6$. Realkalinization of the cells was induced by addition of NaCl (74 mM Na^+) in the presence of 5 mM sodium valerate or sodium butyrate without or with 0.5 mM KCN. Recordings were performed at constant pH_e (7.30) at room temperature. Lower figure: H^+ effluxes expressed as means ± S.E. percentage of control (74 mM Na^+; without amiloride).

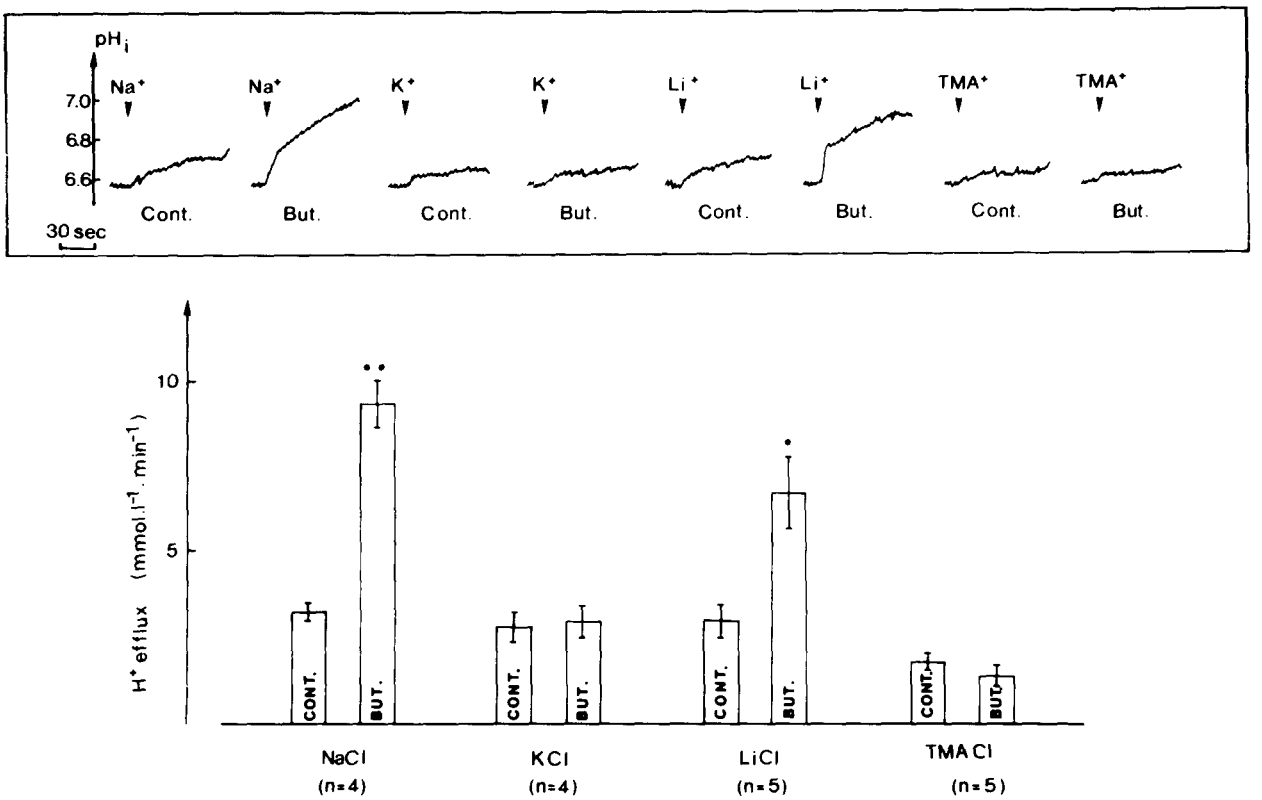


Fig. 3. Effect of different cations on the proton efflux induced by butyrate stimulation. Inset: typical fluorescence traces of BCECF acid-loaded cells. Isolated cells were acidified by the nigericin technique to $pH_i \approx 6.6$. Realkalinization of the cells was induced by addition of different cation chlorides (Na^+, K^+, Li^+, TMA^+, 74 mM) in the absence (Cont.) or the presence of 5 mM sodium butyrate (But.) with 1 mM amiloride. Recordings were performed at constant pH_e (7.30) at room temperature. Lower figure: representation of the calculated rate of proton efflux in the absence (Cont.) or the presence (But.) of 5 mM sodium butyrate, (with 1 mM amiloride. Results are means ± S.E. of n experiments. * $P < 0.02$, ** $P < 0.001$; significantly different from control.

results of these experiments are summarized in Fig. 3. The maximal effect of butyrate was obtained with Na^+. Li^+ also promoted a significant alkalinization with butyrate, but K^+ or TMA^+ were totally ineffective.

Dependence on extracellular sodium or lithium concentrations

In these experiments, acidified BCECF-loaded cells were resuspended in medium containing different sodium or lithium concentrations (20–140 mM) isoosmotically balanced with choline chloride. The results concerning sodium are reported in Fig. 4A and those concerning lithium in Fig. 4B. In the absence of butyrate, the activation of the H^+ efflux by external Na^+ or Li^+ follows a rectangular hyperbola compatible with Michaelis-Menten-type kinetics (data not given) as previously reported for Na^+ [17]. Addition of amiloride to 1 mM to the incubation medium blocked the stimulatory effect of the Na^+ or Li^+ gradient on the H^+ efflux. These data conform with the properties of the Na^+/H^+ antiporter [17] and indicated that Li^+ could be an alternative substrate for the exchanger [5,17,20].

When the cells were exposed to 5 mM sodium butyrate, the Na^+- or Li^+-dependent H^+ efflux was significantly increased at all cation concentrations tested. The effect persists in cells preincubated with 1 mM amiloride, which shows that the Na^+/H^+ antiporter is not involved in the butyrate-induced Na^+- or Li^+-dependent H^+ efflux. For further analysis of the results, the butyrate-insensitive component was subtracted

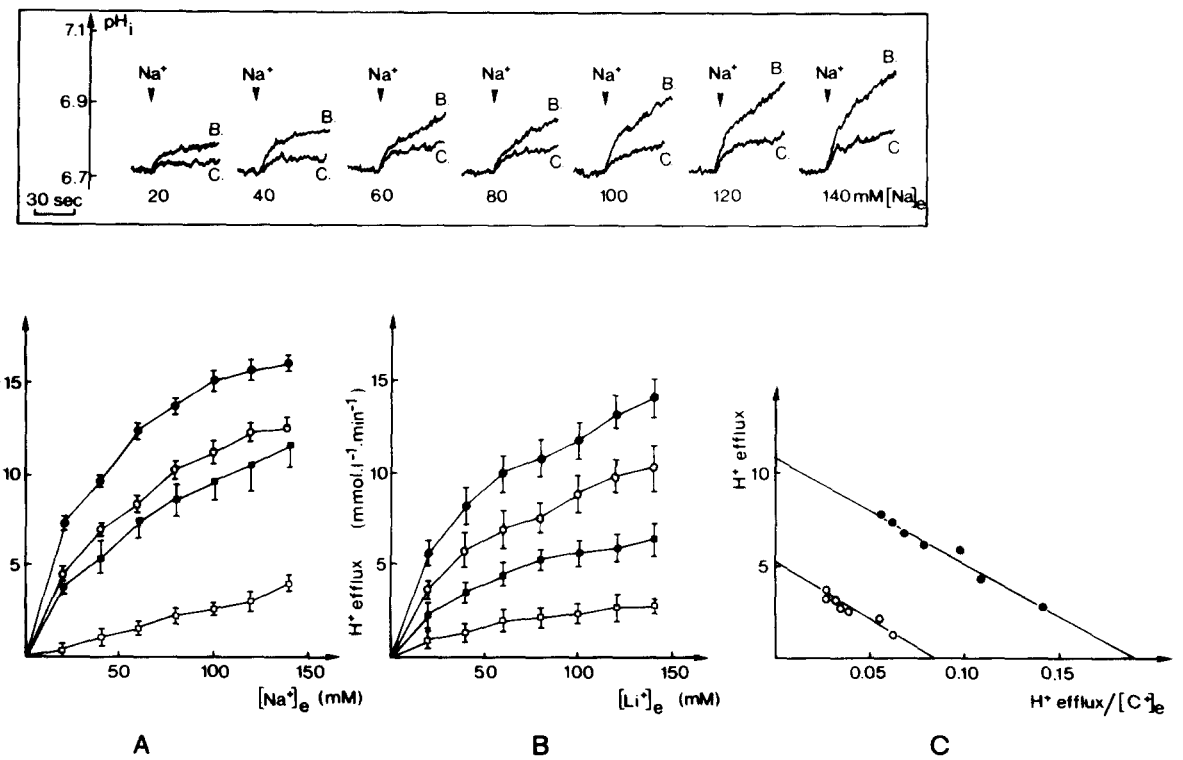


Fig. 4. Effect of external sodium and lithium concentration on the proton efflux induced by butyrate. Inset: typical fluorescence traces of BCECF acid-loaded cells. Effect of different concentrations of Na_e^+ (20–140 mM). Isolated cells were acidified by the nigericin technique to $pH_i \approx 6.7$. Realkalinization of the cells was induced by addition of sodium chloride in the absence (C) or the presence (B) of 5 mM sodium butyrate. Recordings were performed at constant pH_e (7.30) at room temperature. Lower figure: (A) representation of the calculated rate of proton efflux as a function of $[Na^+]_e$ (20–140 mM) in the absence (open symbols) or the presence (closed symbols) of 5 mM butyrate, with (□, ■, $n = 3$) or without (○, ●, $n = 4$) 1 mM amiloride; (B) representation of the calculated rate of proton efflux as a function of $[Li^+]_e$ (20–140 mM) in the absence (open symbols) or the presence (closed symbols) of 5 mM butyrate, with (□, ■, $n = 3$) or without (○, ●, $n = 4$) 1 mM amiloride; (C) Eadie-Hofstee linearization of the butyrate stimulation component for Na^+- and Li^+-dependent proton effluxes. Closed symbols, Na^+; open symbols, Li^+.

from the butyrate-dependent H^+ efflux in the presence of 1 mM amiloride. Eadie-Hofstee plots (V vs. V/S) for H^+ efflux were linear (Fig. 4C) and gave values of V_{max} and K_m of 10.94 mmol $H^+ \cdot l^{-1} \cdot min^{-1}$ and 57.3 mM, respectively in the case of Na^+, and 5.30 nmol $H^+ \cdot l^{-1} \cdot min^{-1}$ and 61.8 mM, respectively, in the case of Li^+.

Dependence on extracellular butyrate concentration

Acidified BCECF-loaded cells were resuspended in 1 mM amiloride medium containing different concentrations of sodium butyrate (from 0.2 to 5 mM). After a 15–30 s equilibration period, 74 mM of Na^+ were added, and changes in fluorescence recorded. The H^+ efflux in the absence of butyrate was measured in each of the five experiments (2.56 ± 0.14 mmol $H^+ \cdot l^{-1} \cdot min^{-1}$) and substracted from the total H^+ efflux. The results are reported in Fig. 5. Na^+-dependent H^+ efflux was a saturable function of the butyrate concentration (Fig. 5A). The Eadie-Hofstee plots of the data, however, were not linear (Fig. 5B).

Dependence on external and internal pH

The effect of pH_e on the Na^+-dependent H^+ efflux induced by butyrate was tested by setting the pH_i at 6.4 and varying the pH_e between 6.4 and 7.6 with 1 M Mes or 1 M Tris solutions. All experiments were conducted in the presence of 1 mM amiloride. The inset of Fig. 6 shows different fluorescent traces obtained with different pH_e values. As expected, the rate of the sodium-induced

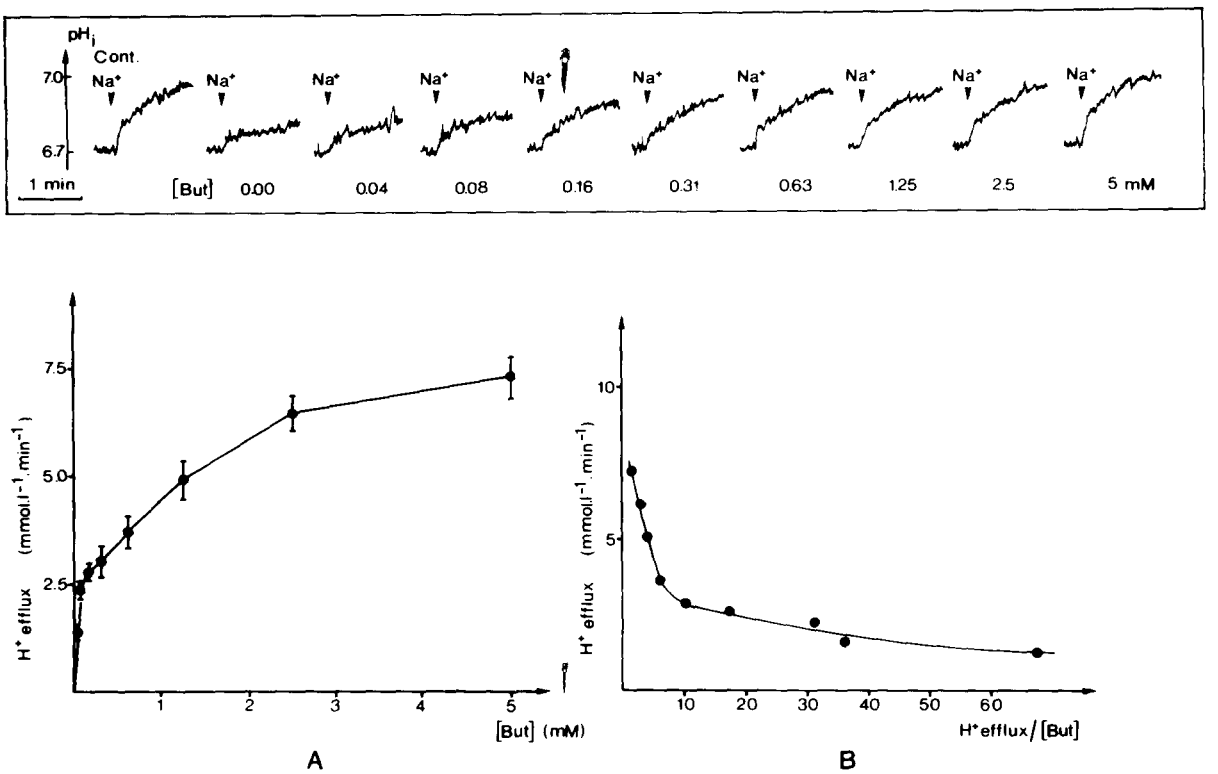


Fig. 5. Effect of external butyrate concentration on Na^+-dependent proton efflux. Inset: typical fluorescence traces of BCECF acid-loaded cells. Isolated cells were acidified by the nigericin technique to $pH_i \approx 6.7$. Realkalinization of the cells was induced by addition of NaCl (74 mM Na^+) in the presence of 0–5 mM sodium butyrate, without (Cont.) or with 1 mM amiloride. Recordings were performed at constant pH_e (7.30) at room temperature. Lower figure: (A) representation of the calculated rate of proton efflux as a function of sodium butyrate concentration (0.04–5 mM) in presence of 1 mM amiloride. Each point represents the mean ± S.E. of five experiments. (B) Eadie-Hofstee linearization of the data from (A).

proton efflux was higher when 5 mM butyrate was added to the incubation medium (Fig. 6A). The differences were statistically significant at all pH_e values tested. The data were then analysed by plotting H^+ efflux against the external OH^- concentration ($[OH^-]_e$). Fig. 6B shows that the proton efflux increased proportionally to $[OH^-]_e$ according to Michaelis-Menten kinetics. The butyrate-independent component of the H^+ efflux was substracted for the total efflux, and Hanes-Woolf linearization was used to calculate the kinetic constants (Fig. 6C). The maximal rate of butyrate-induced Na^+-dependent H^+ efflux was 6.71 ± 0.53 mmol $H^+ \cdot l^{-1} \cdot min^{-1}$ ($n = 3$), with a half-maximal rate at a pH_e of 7.25 ± 0.03 ($n = 3$).

The influence of intracellular pH on the Na^+-dependent H^+ efflux induced by butyrate was studied at a pH_e of 7.2. The BCECF-loaded cells were acidified as described in Materials and Methods and the pH_i was set at various values between 6.55 and 6.95. Alkalinization rate was determined in the presence of 74 mM Na_e^+ in 1 mM amiloride solutions. We plotted the mean of the proton effluxes against the mean of pH_i at 0.1 pH unit intervals. Fig. 7A gives the mean of three preparations. The rate of sodium-induced proton efflux increases in the presence of 5 mM butyrate. After substracting the butyrate-independent component of H^+ efflux, data were analyzed by plotting H^+ efflux against H_i^+ concentration (Fig. 7B) and then by using the Hanes-Woolf representation (Fig. 7C). In the range of pH_i studied, it appeared that the relationship between H_i^+ and the butyrate-induced sodium-dependent H^+ efflux followed simple Michaelis-Menten kinetics with a maximal exchange rate of 6.73 mmol $H^+ \cdot l^{-1} \cdot$

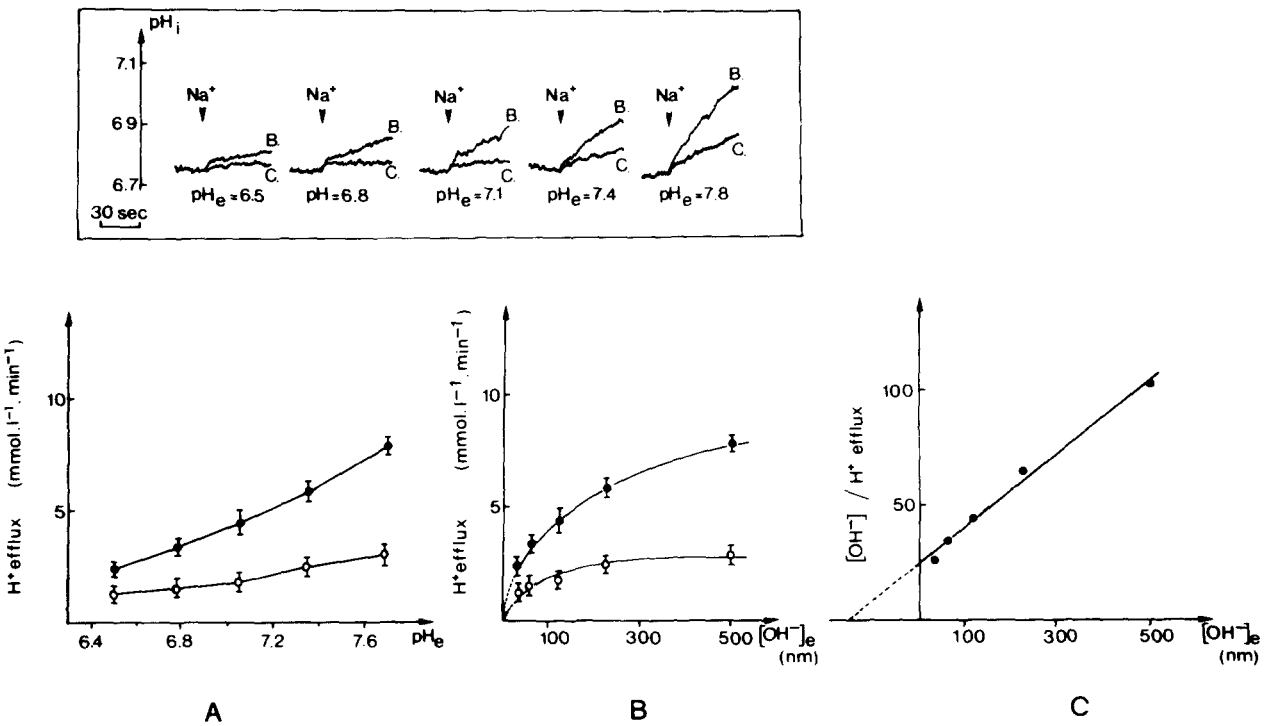


Fig. 6. Effect of external pH on the Na^+-dependent proton efflux induced by butyrate. Inset: typical fluorescence traces of BCECF acid-loaded cells; effect of varying pH_e. Isolated cells were acidified by the nigericin technique to $pH_i \approx 6.75$. Realkalinization of the cells was induced by addition of sodium chloride (74 mM Na^+) in the absence (C) or the presence (B) of 5 mM sodium butyrate, with 1 mM amiloride. Recordings were performed at room temperature. Lower figure: (A) Representation of the calculated rate of proton efflux as a function of pH_e in the absence or the presence of 5 mM butyrate, with 1 mM amiloride. pH_e was modified by addition of 1 M Mes or 1 M Tris between 6.45 and 7.65. Each point represents the mean ± S.E. of three experiments; ●, butyrate; ○, control. (B) Representation of the calculated rate of proton efflux as a function of $[OH^-]_e$ in the absence or the presence of 5 mM butyrate with 1 mM amiloride; ●, butyrate, ○, control. (C) Hanes-Woolf linearization of the data of (B) for butyrate stimulation component.

min^{-1} and a half-maximal rate reached at pH_i of 6.43.

Effect of butyrate on ^{22}Na uptake

The uptake of ^{22}Na in isolated proximal cells at different concentrations of sodium in the presence or the absence of 5 mM butyrate was determined in 1 mM amiloride solutions. In previous studies, we showed that from 1.5 to 20 mM Na_e^+, the intracellular uptake of ^{22}Na is linear for the first minute of incubation [17]. In the present experiments, uptake was therefore taken at 30 s to represent the initial rate of Na^+ entry. Fig. 8 illustrates the uptake of ^{22}Na at two different pH_e values in normal pH_i cells (pH_i 7.2). For both pH_e values, the addition of 5 mM butyrate significantly increased the ^{22}Na uptake. However the

TABLE I

EFFECT OF DIDS ON THE Na^+-DEPENDENT PROTON EFFLUX INDUCED BY THREE SUBSTRATES

The data show the mean ± S.E. for n cell preparations. Results are expressed relative to control uptakes measured in the absence of the inhibitor. BCECF-loaded cells were acidified by the nigericin technique. Realkalinization of the cells was induced by addition of NaCl (74 mM Na^+). The effect of butyrate, lactate or β-hydroxybutyrate as 5 mM sodium salts was tested in the presence of 1 mM amiloride with or without 1 mM DIDS. DIDS was dissolved in dimethyl sulfoxide; recordings were performed with constant pH_e (7.30) at room temperature.

Sodium salt	n	Inhibition (%)
Butyrate	7	32.5 ± 3.7
β-Hydroxybutyrate	4	88.2 ± 6.8
Lactate	5	89.2 ± 9.8

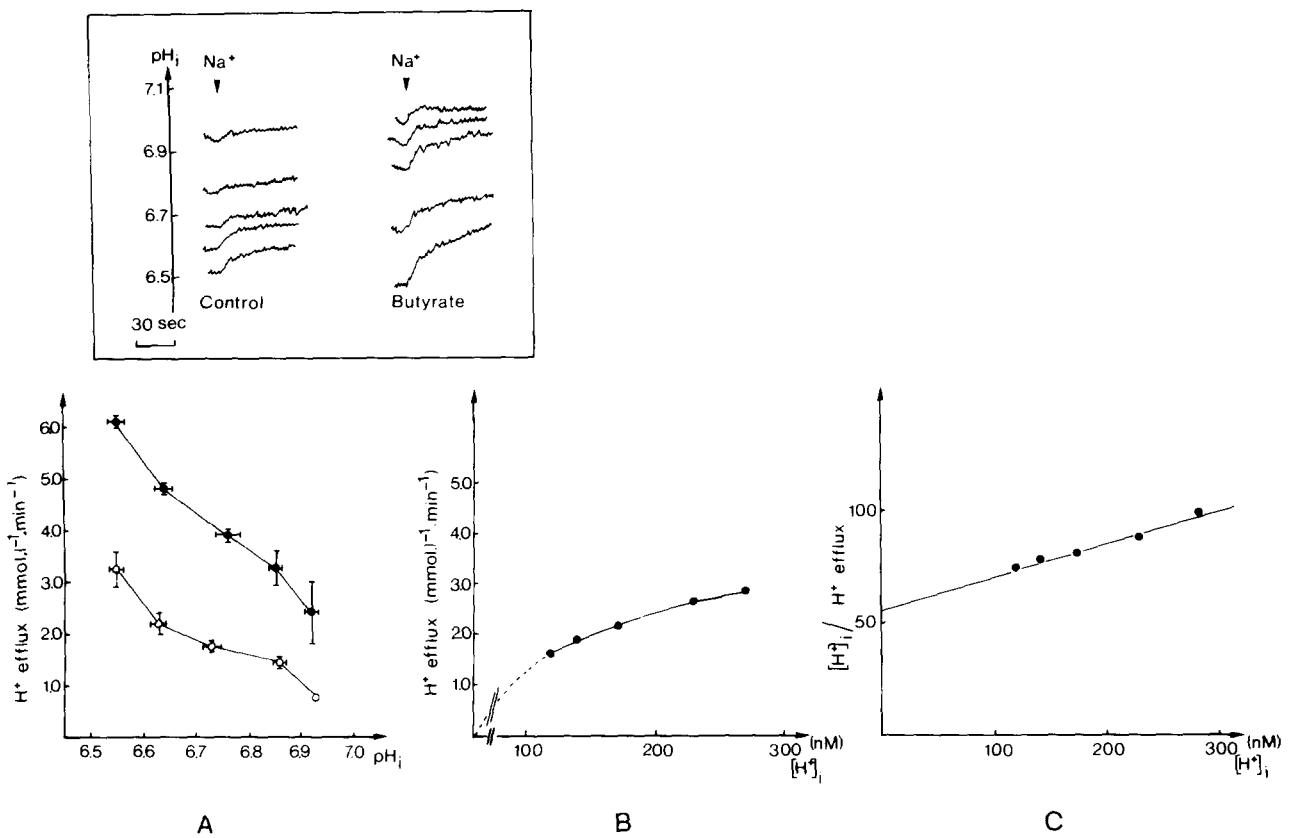


Fig. 7. Effect of internal pH on the Na^+-dependent proton efflux induced by butyrate. Inset: typical fluorescence traces of BCECF acid-loaded cells. Isolated cells were acidified by the nigericin technique at different pH_i levels. Realkalinization of the cells was induced by addition of sodium chloride (74 mM Na^+) in the absence or the presence of 5 mM butyrate, with 1 mM amiloride. Recordings were performed at constant pH_e (7.30) at room temperature. Lower figure: (A) Representation of the calculated rate of proton efflux as a function of pH_i in the absence or the presence of 5 mM butyrate with 1 mM amiloride. Each point represents the mean ± S.E. of 3 experiments; ●, butyrate; ○, control. (B) Representation of the calculated rate of proton efflux as a function of $[H^+]_i$ of butyrate stimulation component; (C) Hanes-Woolf linearization of the data of (B).

effect of butyrate was much more pronounced when the external pH was set at 7.4 than when it was at 6.8.

Effect of different metabolic substrates on Na^+-dependent H^+ efflux

Experiments were first done to study the effects of various monocarboxylates on the Na^+-dependent H^+ efflux. BCECF-loaded cells were acidified by the nigericin technique and the alkalinization rate was determined in 1 mM amiloride solutions containing 5 mM of various monocarboxylates as sodium salts. Experiments were also performed to test the effects of malate, glutamate (5 mM sodium salt) and glucose (5 mM) in the same experimental conditions. The results are shown in Fig. 9. The H^+ efflux with formate was not significantly different from the control value. The rate of alkalinization appeared to increase with the length of the straight chain (acetate < propionate < butyrate < valerate). Lactate and β-hydroxybutyrate, which possess a hydroxyl substituted in Cα and Cβ, respectively, also increased the H^+ efflux, but the amplitude of the response was less than that with acetate. Pyruvate, which has ketone substitution at the Cα position, was without a significant effect on the H^+ efflux. Similarly, the dicarboxylate malate did not cause any significant variation on the H^+ extrusion; this was also the case for glutamate or D-glucose.

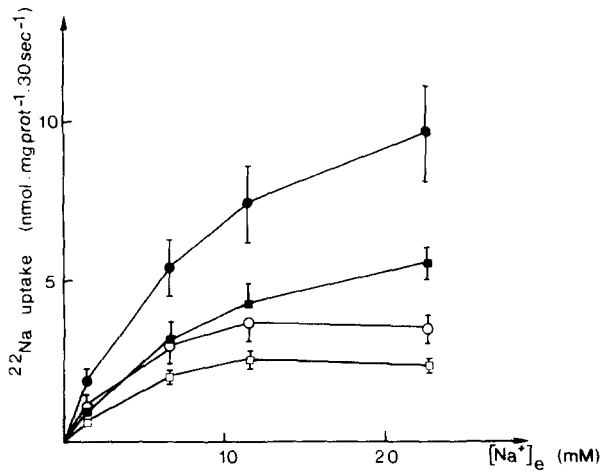
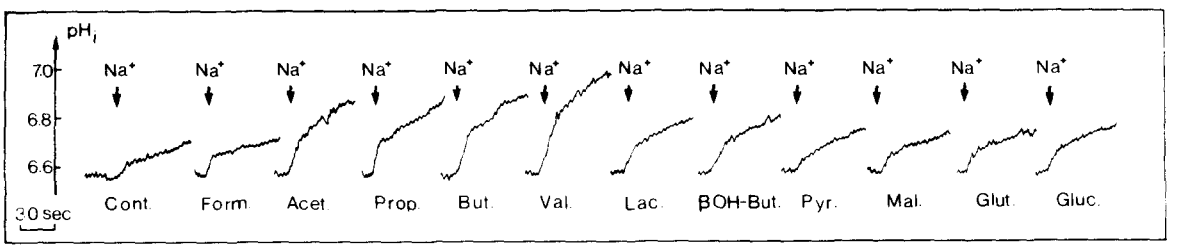


Fig. 8. Effect of butyrate on sodium uptake by isolated proximal cells. The ^{22}Na uptake into $5 \cdot 10^6$ cells suspended in mannitol buffer with (closed symbols) or without (open symbols) 1.5 mM butyrate in the presence of 0.5 mM amiloride was measured. The effect of butyrate at different external sodium concentrations (1.5, 6.5, 11.5 and 21.5 mM NaCl) was tested at two pH_e values (6.8 and 7.4). Uptake was determined after 30 s at each concentration, and isoosmolarity was adjusted with choline chloride. Recordings were performed at 37 °C. Each point represents the mean ± S.E. of four experiments; circles, pH_e 7.4; squares, pH_e 6.8.



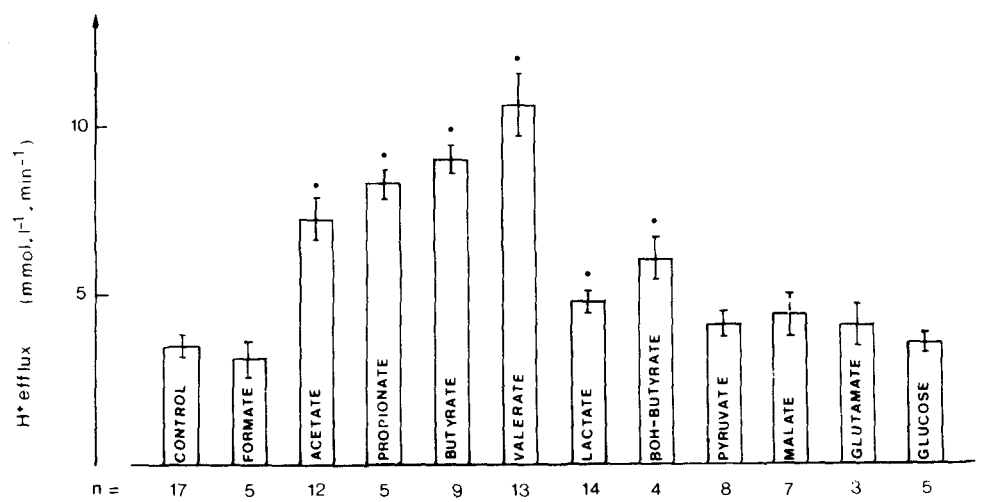


Fig. 9. Effect of different metabolic substrates on the Na^+-dependent proton efflux. Inset: typical fluorescence traces of BCECF acid-loaded cells. Effect of different metabolic substrates. Isolated cells were acidified by the nigericin technique to $pH_i \approx 6.6$. Realkalinization of the cells was induced by addition of sodium chloride (74 mM Na^+) in the presence of various sodium salts of monocarboxylates with 1 mM amiloride. Recordings were performed with constant pH_e (7.30) at room temperature. Lower figure: representation of the calculated rate of proton efflux in the absence (Control) or the presence of various monocarboxylates at a concentration of 5 mM with 1 mM amiloride. Results are the mean ± S.E. of (n) experiments. * $P < 0.001$, significantly different from control.

Effect of DIDS on the Na^+-dependent H^+ efflux induced by the monocarboxylates

The effect of butyrate, lactate and β-hydroxybutyrate on pH_i recovery of acidified cells was studied in the presence or absence of 1 mM DIDS. The inhibitor was added just before the addition of the monocarboxylates. The results of such experiments are reported in Table I. Only one-third of the alkalinization induced by butyrate was blocked by DIDS. The strong inhibition by DIDS of the lactate- or β-hydroxybutyrate-induced alkalinization was noteworthy.

Discussion

The validity of the BCECF fluorimetric technique for measuring intracellular pH in epithelial cells has been discussed in detail in preceding publications [17,21–24]. The method used for cell preparation which avoids any chelating or proteolytic treatment has already been described [14]. Enzymatic and morphological characteristics of cells thus prepared clearly indicate that the cells are mainly proximal [14]. In the present study, we have examined the role of monocarboxylic acid transport on the regulation of pH_i in isolated proximal cells. The addition of 5 mM butyrate to the incubation medium of nigericin acid-loaded cells caused the pH_i to rise rapidly. This effect was dependent on the external concentration of sodium but was insensitive to amiloride, indicating that proton transfer was not through the Na^+/H^+ antiporter. Moreover, the butyrate effect was independent of the intracellular metabolism, since it persisted after preincubation of the cells with KCN. Butyrate also induced a significant increase in the Na^+ influx. It is therefore probable that, as previously proposed, the initial mechanism underlying H^+ extrusion in the presence of butyrate is a Na^+-coupled adsorption of monocarboxylate. Nord et al. [25] made a detailed study of the specificity of Na^+-dependent monocarboxylic acid transport in renal brush-border membranes. They reported that the monocarboxylate carrier interacts with acetate, propionate, butyrate and valerate. The present data corroborate their results. The efficiency of the organic anion to promote pH_i recovery increases with the length of the straight unsubstituted chain (acetate < propionate < butyrate < valerate). This corresponds with the increase of lipidic non-ionic diffusion reported by Walter et al. [26] in lipid bilayer membranes. The whole mechanism of H^+ extrusion is compatible with Na^+-coupled organic anion transport followed by non-ionic diffusion of the undissociated organic acid from the cells to the incubation medium. In our experiments, formate did not significantly modify the pH_i recovery of acidified cells. This organic anion is probably not transported by the monocarboxylate carrier [25] and the ionic form (in excess at pH_e 7.3) does not permeated the cellular membrane freely. L-Lactate enhanced the proton efflux, but in spite of its high affinity for the Na^+ cotransporter, it is less efficient than β-hydroxybutyrate. Furthermore, pyruvate, which is also cotransported with a good affinity, remained without significant effect on pH_i recovery. Taken together, these results indicate that the first condition for the realkalinization induced by organic acid is the existence of a Na^+ cotransport via the monocarboxylate carrier. However, when the affinities for the carrier are identical, the limiting factor becomes the lipidic permeability of the non-ionic form. This permeability is decreased when there is an hydroxyl or a ketone substitution on the straight carbon chain [25]. Finally, acetate, propionate, butyrate and valerate, which possess both a high affinity for the carrier [25] and a high lipophilic permeability of their non-ionic form [26,27], represent the ideal substrates for ensuring a proton extrusion from acidified cells.

Using butyrate as substrate, we have studied some of the physical characteristics of the proton extrusion induced by Na^+-dependent monocarboxylate transport. In the presence of amiloride, the rate of Na^+-dependent H^+ efflux indirectly reflects the activity of the carrier. The relationship between H^+ efflux and butyrate concentration was not a rectangular hyperbola, as would be expected it the kinetics were of a simple Michaelis-Menten type. This suggests that the monocarboxylate induced the H^+ efflux via several pathways or/and that the monocarboxylate was cotransported by at least two different carriers. The latter hypothesis accords with the observations of Nord et al. [28] who showed that pyruvate transport across the renal brush border shares

both monocarboxylic and dicarboxylic acid transport systems. However, these authors also demonstrated that L-lactate was cotransported by a single carrier. Since the relationship between H^+ efflux and the butyrate carrier is very indirect, it is not possible to draw final conclusions.

The V_{max} of the carrier was higher in the presence of Na^+ than in the presence of Li^+, but surprisingly, the apparent K_m for Li^+ was not significantly different from that for Na^+. Although no corresponding data occur in the literature, it has been reported that the affinity of the Na^+-dependent carrier for succinate is 40-fold higher in Na^+ than in Li^+ [29]. Our data indicate that the monocarboxylate carrier may be activated equally well by Li^+ as by Na^+.

The rate of sodium-dependent proton efflux induced by butyrate was higher when the transmembrane H^+ gradient was increased.

The dependence on external and internal pH was symmetric and in both cases the H^+ efflux followed Michaelis-Menten-like kinetics. Over the 6.5 to 7.7 pH_e range, the ratio between the anionic and the protonated forms of butyrate (COO^-/COOH) increased from 50 to 794. At 5 mM external butyrate, however, the cotransported form (COO^-) remained largely dominant at all the pH_e values studied. Modification of the Na^+ cotransport activity by the availability of the anionic form is therefore negligible. Efflux of the protonated form, however, would impose an H^+ efflux obeying to Fick's law rather than saturable kinetics. Another possible mechanism for the induced H^+ efflux is an organic anion/OH^- exchange [8] in which, when the inwardly directed OH^- gradient is high, OH^- would move through the carrier in exchange for internal COO^-.

In our experiments, when the antiporter was blocked by amiloride, the intracytoplasmic fluorescence of acidified cells (resuspended in a medium at pH 7.2) did not vary significantly with time, indicating that proximal cells only possess a very weak passive permeability to both H^+ and OH^-. The addition of butyrate did not modify the proton efflux, but in the presence of Na^+, intracellular butyrate was driven by the Na^+-dependent monocarboxylate carrier. The efflux of the anionic form proceeded against its concentration gradient by means of the anionic exchanger energized by the important outwardly directed OH^- gradient. Under our experimental conditions, the activity of the exchanger would be controlled by the amplitude of the OH^- gradient. The sensitivity to inhibition by DIDS of the H^+ efflux induced by monocarboxylates is consistent with the existence of the anion/OH^- exchanger [13,30]. However, the alkalinization obtained with butyrate was only partially inhibited by DIDS. This indicates that the major part of the H^+ efflux driven by the short-chain fatty acids resulted from a process of non-ionic diffusion. Conversely, in the case of lactate and β-hydroxybutyrate, it seems that the major route for H^+ extrusion was via an anion exchange pathway.

In conclusion, acidified cells are able to regulate their intracellular pH independently of Na^+/H^+ antiporter activity. The process responsible for H^+ efflux induced by butyrate in the presence of external Na^+ may include non-ionic diffusion of weak acids and organic anion/OH^- exchange. These two mechanisms are dependent on the activity of the Na^+ cotransport of monocarboxylates modulating the intracellular anionic form.

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