Anion-dependent Mg$^{2+}$ influx and a role for a vacuolar H$^+$-ATPase in sheep ruminal epithelial cells

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Schweigel, Monika and Holger Martens. Anion-dependent Mg$^{2+}$ influx and a role for a vacuolar H$^+$-ATPase in sheep ruminal epithelial cells. Am J Physiol Gastrointest Liver Physiol 285: G45–G53, 2003. First published February 26, 2003; 10.1152/ajpgi.00396.2002.—The K$^+$-insensitive component of Mg$^{2+}$ influx in primary culture of ruminal epithelial cells (REC) was examined by means of fluorescence techniques. The effects of extracellular anions, ruminal fermentation products, and transport inhibitors on the intracellular free Mg$^{2+}$ concentration ([Mg$^{2+}$]i), Mg$^{2+}$ uptake, and intracellular pH were determined. Under control conditions (HEPES-buffered high-NaCl medium), the [Mg$^{2+}$]i of REC increased from 0.56 ± 0.14 to 0.76 ± 0.06 mM, corresponding to a Mg$^{2+}$ uptake rate of 15 μM/min. Exposure to butyrate did not affect Mg$^{2+}$ uptake, but it was stimulated (by 84 ± 19%) in the presence of CO$_2$/HCO$_3$$. In contrast, Mg$^{2+}$ uptake was strongly diminished if REC were suspended in HCO$_3$-buffered high-KCl medium (22.3 ± 4 μM/min) rather than in HEPES-buffered KCl medium (37.5 ± 6 μM/min). After switching from high- to low-Cl$^-$ solution, [Mg$^{2+}$]i was reduced from 0.64 ± 0.09 to 0.32 ± 0.16 mM and the CO$_2$/HCO$_3$-stimulated Mg$^{2+}$ uptake was completely inhibited. Bumetanide and furosemide blocked the rate of Mg$^{2+}$ uptake by 64 and 40%, respectively. Specific blockers of vacuolar Mg$^{2+}$-ATPase reduced the [Mg$^{2+}$]i (36%) and Mg$^{2+}$ influx (38%) into REC. We interpret this data to mean that the K$^+$-insensitive Mg$^{2+}$ influx into REC is mediated by a cotransport of Mg$^{2+}$ and Cl$^-$ and is energized by an H$^+$-ATPase. The stimulation of Mg$^{2+}$ transport by ruminal fermentation products may result from a modulation of the H$^+$-ATPase activity.

sheep rumen; epithelial cells; intracellular magnesium; Mg$^{2+}$-Cl$^-$ cotransport; mag-fura-2

IN RUMINATING ANIMALS, most of the required Mg$^{2+}$ is absorbed from the forestomach by active, transcellular mechanisms. Uptake of Mg$^{2+}$ into ruminal epithelial cells (REC) has been shown to be mediated by two parallel-working transport mechanisms. Part of the Mg$^{2+}$ influx is K$^+$ sensitive and may represent electrodiffusive Mg$^{2+}$ uptake by an ion channel (35). The greater part (62%) is, however, K$^+$ insensitive, and previous experiments carried out with isolated rumen epithelium and isolated ruminal epithelial cells (REC) in our laboratory have established that ruminal fermentation products [short-chain fatty acids (SCFA), CO$_2$] stimulate transepithelial Mg$^{2+}$ absorption (23) and Mg$^{2+}$ influx into REC (37). Initially, it was assumed that the stimulation of Mg$^{2+}$ transport was via Mg$^{2+}$/H$^+$ exchange in the apical membrane (23), by means of the proton load resulting from the absorption of SCFA in their protonized form and the intracellular hydration of CO$_2$. More direct investigations with isolated REC have provided evidence for a symport of Mg$^{2+}$ with anions and confirmed that SCFA and CO$_2$ activate H$^+$ efflux by the Na$^+$/H$^+$ exchanger (37). A positive relationship between intracellular H$^+$ availability and transepithelial Mg$^{2+}$ transport (23) can also be explained by the existence of an additional Na$^+$-independent acid extrusion process (e.g., an H$^+$ pump) that occurs in the cell membrane of REC and energizes Mg$^{2+}$ uptake. To date, there is no information regarding the existence of such an active H$^+$-extruding mechanism in REC. This therefore is explored in the present work. In addition, we have verified the influence of the predominant ruminal anions [HCO$_3$, dissociated SCFA (SCFA$^-$), and Cl$^-$] on Mg$^{2+}$ uptake.

To this purpose, we have performed experiments with isolated REC. With the aid of the fluorescence probes mag-fura 2 and BCECF, we have measured the intracellular free Mg$^{2+}$ concentration ([Mg$^{2+}$]i) and the intracellular pH (pH$_i$) of REC under basal conditions and after changing the transmembrane chemical gradients for butyrate, CO$_2$/HCO$_3$, and Cl$^-$. To differentiate between K$^+$-sensitive and -insensitive Mg$^{2+}$ transport, some of the experiments have been carried out in high-K$^+$/low-Na$^+$ media. Additionally, transport inhibitors (loop diuretics, chlorothiazide, bafilomycin A$_1$, and folicycin) have been used to examine the possible role of an H$^+$-ATPase in Mg$^{2+}$-anion cotransport.

MATERIALS AND METHODS

Materials. Medium 199, trypsin, glutamine, antibiotics (gentamycin, nystatin, kanamycin), and FCS were purchased from Sigma (St. Louis, MO). Dulbecco’s phosphate-buffered saline (DPBS) and collagen were obtained from Biochrom (Berlin, Germany). Mag-fura-2 AM, BCECF-AM, and pluronic acid were from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma.

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Cell culture. Primary cultures of REC were prepared as described by Galí et al. (9). Briefly, REC were isolated by fractional trypsinization and grown in medium 199 containing 10% FCS, 1.36 mM glutamine, 20 mM HEPES, and antibiotics (gentamycin 50 mg/l, kanamycin 100 mg/l) in an atmosphere of humidified air-5% CO$_2$ at 38°C. Experiments were performed 6–12 days after seeding.

Solutions. The control solutions were HEPES-buffered high-Na$^+$, high-Cl$^-$ solution (in mM: 145 NaCl, 5 KCl, 1 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, 5 glucose, pH 7.4) and HEPES-buffered high-Na$^+$/low-Cl$^-$ solution (in mM: 110 Na-gluc onate, 25 NaCl, 5 KCl, 1 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, 5 glucose, pH 7.4). To inhibit the K$^+$-sensitive part of Mg$^{2+}$ uptake, cells were incubated in a HEPES-buffered high-K$^+$ solution containing (in mM) 15 NaCl, 135 KCl, 1 CaCl$_2$, 2 MgCl$_2$, 10 HEPES, 5 glucose, pH 7.4. In butyrate-containing, HEPES-buffered high-NaCl solution, 20 mM NaCl was replaced by Na-butyrate. To investigate the effect of lowering the extracellular Cl$^-$ concentration ([Cl$^-$]e) and the effect of CO$_2$/HCO$_3$ on Mg$^{2+}$ transport the composition of these solutions were changed. The composition of the modified experimental solution is given in Table 1. All HCO$_3$-containing solutions were preequilibrated with 95% air-5% CO$_2$.

Measurement of cytoplasmic Mg$^{2+}$ and pH by spectrofluorometry. Cells were loaded with either 5 μM mag-fura-2 AM or 0.5 μM BCECF-AM for the determination of [Mg$^{2+}$]i and the effect of CO$_2$/HCO$_3$ on Mg$^{2+}$ uptake. The procedure was repeated for various pH values between 6.0 and 8.0. Statistical analysis. If not otherwise stated, data are presented as means ± SE. Significance was determined by Student’s t-test or Tukey’s analysis of variance as appropriate. Correlations between variables were tested by calculating Pearson’s Product Moment correlation coefficients. P < 0.05 was considered to be significant. All statistical calculations were performed by using SigmaStat software (Jandel Scientific).

RESULTS

Some in vivo and in vitro studies showed an interrelationship between the intracellular supply of H$^+$ and Mg$^{2+}$ transport (7, 23). Therefore, we first studied the effect of various experimental manipulations on basal pH, and pH-regulating mechanisms in REC.

Effect of butyrate, of CO$_2$/HCO$_3$, and of changing the extracellular [K$^+$] and [Cl$^-$] on pH$^i$ of REC. Under control conditions (HEPES-buffered high-NaCl or high-KCl medium), baseline pH$^i$ was 6.83 ± 0.1 and 6.89 ± 0.03, respectively. Thus the resting pH$^i$ measured in the high-K$^+$ medium was not significantly different from that measured in Na$^+$ medium (Fig. 1). In contrast, pH$^i$ decreased to 6.65 ± 0.01 after switching from control solution (HEPES-buffered high-NaCl) to a HEPES-buffered high-Na$^+$/low-Cl$^-$ medium (Fig. 1). Incubation of REC in butyrate- and/or CO$_2$/HCO$_3$-containing solutions led to an intracellular acidification (Fig. 1). Thereupon, REC recovered to near control levels during the experimental period (Fig. 1). Neither increasing the extracellular K$^+$ concentration ([K$^+$]e) (from 5 to 135 mM) nor decreasing the [Cl$^-$]e (from 136/116 to 36 mM) affected the ability of REC to recover from the acid load. On average, the pH$^i$ recovered by 0.19 ± 0.05 pH units within 10 min, but slightly higher recovery rates of 0.24 ± 0.09 and 0.22 ± 0.06 units per 10 min were observed in Cl$^-$-reduced media with butyrate and/or CO$_2$/HCO$_3$, respectively.

Effect of butyrate and CO$_2$/HCO$_3$ on [Mg$^{2+}$]i, and the Mg$^{2+}$ uptake rate of REC incubated in high-NaCl or high-KCl media. Figure 2 shows a comparison of the effects of butyrate and/or CO$_2$/HCO$_3$ on [Mg$^{2+}$]i and Mg$^{2+}$ influx in high-NaCl and -KCl media. The latter was used to eliminate the electrodiffusive K$^+$-sensitive part of Mg$^{2+}$ uptake (22, 35). Resting [Mg$^{2+}$]i, determined in HEPES-buffered control solutions at the beginning of the experiments, was significantly lower in the high-NaCl medium (0.56 ± 0.14 mM) compared with that in high-KCl medium (0.88 ± 0.32 mM). In both media, an increase of [Mg$^{2+}$]i was observed, which led to [Mg$^{2+}$]i levels of 0.76 ± 0.06 mM (high-NaCl medium) and 1.26 ± 0.3 mM (high-KCl medium), respectively. As in our previous study (37), the Mg$^{2+}$

Table 1. Composition of experimental solutions

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<th>High NaCl, HCO$_3$</th>
<th>High Na$^+$, Low Cl$^-$, HCO$_3$</th>
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Values are concentrations in millimoles per liter. Solution pH was adjusted to 7.4 using appropriate buffers. Osmolarity of all solutions was 290–300 mosM.
influx was stimulated by the presence of butyrate and/or CO₂/HCO₃⁻ in high-NaCl solutions. After the 10-min measuring period, the [Mg²⁺]ᵢ was significantly higher (Fig. 2) in REC incubated in media with CO₂/HCO₃⁻ (0.92 ± 0.13 mM) or CO₂/HCO₃⁻ and butyrate (1.05 ± 0.4 mM) compared with that incubated in control medium (0.76 ± 0.06 mM). Additionally, Mg²⁺ uptake rates were stimulated by 84 ± 19 and 93 ± 40% in HCO₃⁻-buffered media without or with butyrate, respectively (Fig. 3). It is important to note that, in the presence of CO₂/HCO₃⁻, neither the [Mg²⁺]ᵢ levels nor the Mg²⁺ uptake rates were significantly changed by the addition of butyrate. Furthermore, butyrate alone was not effective in increasing Mg²⁺ influx (12.8 ± 5 μM/min) compared with HEPES-buffered control medium (15 ± 1 μM/min).

The stimulating effect of CO₂/HCO₃⁻ on Mg²⁺ uptake was fully abolished if REC were incubated in a high-KCl medium, but this negative effect was compensated...
by supplementation of butyrate (Fig. 2 and 4). In the HCO$_3^-$-buffered high-KCl medium, the rate of Mg$^{2+}$ uptake was reduced to 22.3 ± 4 µM/min, which was significantly less than the rate observed in HEPES-buffered K$^+$ medium (37.5 ± 6 µM/min) or in K$^+$ medium with CO$_2$/HCO$_3^-$ and butyrate (41.9 ± 12.4 µM/min) (Fig. 4).

Influence of a reduction of [Cl$^-$_e] on [Mg$^{2+}$]$_i$ and CO$_2$/HCO$_3^-$-stimulated Mg$^{2+}$ uptake. Because, after SCFA$^-$/HCO$_3^-$, Cl$^-$ is the most abundant anion in the ruminal fluid, we then analyzed the role of the [Cl$^-$_e] on [Mg$^{2+}$]$_i$ and on the rate of Mg$^{2+}$ uptake in REC. The cells were suspended in butyrate- and/or CO$_2$/HCO$_3^-$-containing high-Na$^+$ solutions with a [Cl$^-$_e] of 116/136 or 36 mM, respectively.

As shown in Fig. 5, the [Mg$^{2+}$]$_i$ of REC clearly depended on the extracellular Cl$^-$ level. As [Cl$^-$_e] was reduced, the initial [Mg$^{2+}$]$_i$, fell from 0.64 ± 0.09 to 0.32 ± 0.16 mM (medium with CO$_2$/HCO$_3^-$) and from 0.7 ± 0.26 to 0.37 ± 0.2 mM (medium with CO$_2$/HCO$_3^-$ and butyrate), respectively. In all media, an increase of [Mg$^{2+}$]$_i$ was observed, but the [Mg$^{2+}$]$_i$ of REC incubated in the Cl$^-$-reduced media stayed well below that of cells in high-Cl$^-$ media (Fig. 5). These effects of a [Cl$^-$_e] reduction were independent of the presence of butyrate and/or CO$_2$/HCO$_3^-$ in the experimental solutions.

Furthermore, reduction of the [Cl$^-$_e] diminished the rate of Mg$^{2+}$ uptake. On exposure to medium with 36 mM Cl$^-$, the rate of Mg$^{2+}$ uptake dropped from 28 ± 5 to 15 ± 5 µM/min (medium with CO$_2$/HCO$_3^-$-, containing Na$^+$ solutions) and from 29.0 ± 10 to 20 ± 5 µM/min (CO$_2$/HCO$_3^-$- and butyrate-containing Na$^+$ solutions). Figure 3 illustrates that the CO$_2$/HCO$_3^-$-dependent stimulation of Mg$^{2+}$ uptake is completely abolished after the reduction of [Cl$^-$_e]. Butyrate, on the other hand, can partly substitute for extracellular Cl$^-$ under these experimental conditions (Fig. 3). As a result, the Mg$^{2+}$ uptake rate was increased by 38 ± 18% compared with control values (in HEPES-buffered high-NaCl medium).

To show that the observed effects were induced by the decrease of [Cl$^-$_e], not by disturbing the content of other ions (HCO$_3^-$, Na$^+$) in the medium, we performed control experiments with gluconate as a substitute for Cl$^-$. The [Cl$^-$_e] of the solutions used in these experiments were replaced by reducing 100 mM (CO$_2$/HCO$_3^-$-containing solutions) or 80 mM (CO$_2$/HCO$_3^-$ and butyrate-containing solutions) of Cl$^-$ by gluconate, leaving the concentration of all other ions unchanged. Under these conditions the same marked decrease of the [Mg$^{2+}$]$_i$ was seen (results not shown). Furthermore, the Mg$^{2+}$ uptake rate was reduced from 37 ± 5 to 19 ± 1 µM/min (high-Na$^+$ solution with CO$_2$/HCO$_3^-$) and from 31.5 ± 5 to 22 ± 4 µM/min (high-Na$^+$ solution with CO$_2$/HCO$_3^-$ and butyrate), respectively.

Effect of known inhibitors of cation-Cl$^-$ cotransporters on [Mg$^{2+}$]$_i$ and pH$_i$. The loop-diuretics furosemide and bumetanide have been shown to inhibit anion-dependent electroneutral Mg$^{2+}$ uptake in Yoshida ascites tumor cells (14). For this reason, we tested the effect of these inhibitors in a subsequent series of experiments. REC were suspended in high-K$^+$ medium (to abolish the membrane potential ($E_m$)) with CO$_2$/HCO$_3^-$ and butyrate, and the [Mg$^{2+}$]$_i$, was determined over a 10-min period. Compared with control conditions, we found a strong reduction of the [Mg$^{2+}$]$_i$ after application of 100 µM furosemide or bumetanide, respectively. As shown in Fig. 6, control cells had an initial [Mg$^{2+}$]$_i$ of 0.87 ± 0.12 mM, but this was only 0.55 ± 0.05 and 0.32 ± 0.09 mM in REC treated with 100 µM furosemide or bumetanide, respectively. Despite an increase of the [Mg$^{2+}$]$_i$, in all experimental conditions.
In contrast, basal pHi and the ability of REC to recover from the acid load was not influenced by the presence of bumetanide in the extracellular solution. The thiazide-type diuretic chlorothiazide was not effective in blocking Mg\(^{2+}\) uptake (Fig. 6) but increased basal pHi and stimulated the acid-induced pH\(_i\) recovery (results not shown).

So far, the results confirm our previous conclusion (37) that the so-called K\(^+\)-insensitive Mg\(^{2+}\) uptake mechanism is anion dependent and indicate a symport of Mg\(^{2+}\) with Cl\(^-\). However, such a cotransport offers no direct explanation for the positive effects of SCFA and CO\(_2\) on Mg\(^{2+}\) transport. Our next hypothesis was that they activate a mechanism that increases the driving force for Mg\(^{2+}\)-Cl\(^-\) cotransport and perhaps also for electrodiffusive Mg\(^{2+}\) influx. Because it seems from in vitro experiments with isolated epithelia that there is a coupling of H\(^+\) secretion and Mg\(^{2+}\) transport (23), we have tested the possibility that an H\(^+\)-ATPase is involved in Mg\(^{2+}\) uptake.

Is there a role for an H\(^+\) pump in Mg\(^{2+}\) transport? To this purpose, we performed experiments with specific blockers of vacuolar H\(^+\)-ATPases, namely bafilomycin A\(_1\) and foliomycin.

First, the effect of bafilomycin A\(_1\) (5 \(\mu M\)) on the free cytosolic [Mg\(^{2+}\)] of REC incubated in a butyrate-containing, high-NaCl medium was examined. As shown in Fig. 7, bafilomycin A\(_1\) led to a reduction of the [Mg\(^{2+}\)]\(_i\) of REC. The [Mg\(^{2+}\)]\(_i\), determined at the beginning of the measuring period (after a 5-min preincubation with or without inhibitor) was 0.76 \(\pm\) 0.04 mM in control cells and 0.49 \(\pm\) 0.08 mM in bafilomycin-A\(_1\)-treated cells. After a further 10-min period, the [Mg\(^{2+}\)]\(_i\) of nontreated cells was increased to 0.83 \(\pm\) 0.05 mM, compared with 0.59 \(\pm\) 0.07 mM in REC exposed to the inhibitor.

Moreover, with the more specific inhibitor foliomycin (2 \(\mu M\)), we found a significant reduction of the rate of Mg\(^{2+}\) uptake (Fig. 8). REC incubated in the CO\(_2\)/HCO\(_3\)-containing high-NaCl solution took up Mg\(^{2+}\) at a rate of 47 \(\pm\) 1 \(\mu M/\text{min}\), but after application of foliomycin, the uptake rate was reduced to 30 \(\pm\) 3 \(\mu M/\text{min}\), which corresponded to a 36% decrease.

With both bafilomycin A\(_1\) and foliomycin, the initial pH\(_i\) \((-0.05 \pm 0.01; -0.074 \pm 0.05\) and the rate of pH\(_i\) recovery \((0.04 \pm 0.01; -0.07 \pm 0.02)\) was reduced, compared with control values.

**DISCUSSION**

**Modulation of pH\(_i\) by CO\(_2\)/HCO\(_3\), butyrate, [K\(^+\)], and [Cl\(^-\)]**. In a previous study (37), we have shown that there is no direct coupling between H\(^+\) efflux and Mg\(^{2+}\) influx. However, there is a possibility that the pH\(_i\) may have indirect effects on Mg\(^{2+}\) transport. Therefore, we have tested the effects of various experimental manipulations on pH\(_i\). Changes from solutions containing HEPES to those containing CO\(_2\)/HCO\(_3\) and/or butyrate led to intracellular acidification, which can be explained partly by the permeation of the very lipidsoluble CO\(_2\) and/or the protonated form of the fatty acid across the cell membrane. Furthermore, with an extracellular pH (pH\(_e\)) of 7.4 most of the butyrate (dissociation constant \(\sim 4.8\)) is present in dissociated form. Entry of SCFA\(^-\) into REC is mediated by an ion...
exchange for intracellular HCO$_3^-$ (21), thereby reducing the intracellular buffer capacity. Consequently, the pH$_i$ of REC decreases to a greater extent in butyrate- than in CO$_2$/HCO$_3^-$-containing solutions. The CO$_2$- and/or butyrate-induced acidification rapidly activates cell-alkalinizing mechanisms, e.g., Na$^+$/H$^+$ exchanger and Na$^+$/HCO$_3^-$ symport (27, 37) leading to pH$_i$ recovery. The rate of recovery of pH$_i$ seen in this study (0.17–0.24 pH units within 10 min) is consistent with our earlier data (37) and with results from the only other study dealing with pH$_i$ regulation in REC (27). Neither an increase of [K$^+$]$_e$ (from 5 to 135 mM) nor a decrease of [Cl$^-$]$_e$ (from 116/136 to 36 mM) reduces the ability of REC to recover from an acid load. The pH$_i$ recovery rate is slightly higher in a medium with a reduced Cl$^-$ content. Although the reason for this effect has not been investigated in detail, it can be attributed to an inhibition of the activity of the HCO$_3^-$/Cl$^-$ exchanger, which is present in the cell membrane of REC (24). Such an effect has been described repeatedly in other cell systems (15, 20) and is confirmed by the finding that pH$_i$ of REC is reduced in HEPES-buffered low-Cl$^-$ medium.

Interrelationship between pH$_i$ acidification and [Mg$^{2+}$]$_i$. Some reports suggest a pH$_i$-induced elevation of [Mg$^{2+}$]$_i$ resulting from release of Mg$^{2+}$ from intracellular compounds. In our study, proton loads evoked by switching from HEPES-buffered to CO$_2$/HCO$_3^-$- and/or butyrate-containing high-NaCl solutions led to marked pH$_i$ acidification but did not significantly alter the basal [Mg$^{2+}$]$_i$ of REC. This corresponds well to the existence of REC showing spontaneously low pH$_i$ values of 6.4 ± 0.08, but basal [Mg$^{2+}$]$_i$ levels (0.67 ± 0.09 mM) not different from those of normal REC (0.7 ± 0.05 mM; results not shown). Likewise, the elevation in [Mg$^{2+}$]$_i$ observed during the time course of our experiments is not simply linked to a decrease in pH$_i$. If it were, the effective pH$_i$ regulation back to near basal values should normalize [Mg$^{2+}$]$_i$. This was not the case, and, with the same or even stronger levels of acidification, the increase in [Mg$^{2+}$]$_i$ is significantly reduced if REC were incubated in high-NaCl solutions with only butyrate, in Cl$^-$-reduced solutions, or in high-KCl medium with CO$_2$/HCO$_3^-$. These results suggest that REC, like other epithelial cells (30), regulate or stabilize their [Mg$^{2+}$]$_i$ independently of the pH$_i$ or by means not directly related to pH$_i$. Although the mechanisms have to be determined in detail, it seems that the Na$^+$/Mg$^{2+}$ exchanger, which we have shown to exist in the cell membrane of REC (37), contributes to this process.

Role of Na$^+$/Mg$^{2+}$ exchanger. On the other hand, it can be excluded that changes in the Na$^+$/Mg$^{2+}$ exchanger activity are responsible for the [Mg$^{2+}$]$_i$ increase seen in this study. Most of our experiments have been performed in high-Na$^+$ media (extracellular Na$^+$ concentration, 75–145 mM) with an [Mg$^{2+}$]$_i$ of 2 mM. Under such conditions, the Na$^+$/Mg$^{2+}$ exchanger always operates in the forward mode, thereby mediating Mg$^{2+}$ efflux and Na$^+$ uptake (36). Therefore, its activity should result in an [Mg$^{2+}$]$_i$ decrease rather than in the increase seen in our study. The latter effect would require an inhibition or reduced activity of the Na$^+$/ Mg$^{2+}$ exchanger. This conclusion is supported by results from preliminary experiments with the nonspecific inhibitor amiloride. Application of amiloride in a low dosage of 100 µM led to the expected sharp increase in [Mg$^{2+}$]$_i$ (results not shown). Furthermore, the higher [Mg$^{2+}$]$_i$, starting levels measured in the high-K$^+$/low-Na$^+$ (135/15 mM) media are partly explicable by a reduced activity of the Na$^+$/Mg$^{2+}$ exchanger resulting from the lowering of the Na$^+$ gradient across the cell membrane. It also explains the persistence of an “apparent” Mg$^{2+}$ uptake after inhibiting K$^+$-sensitive and -insensitive components of Mg$^{2+}$ influx by incubation of REC in high-KCl media with either high HCO$_3^-$, furosemide, or bumetanide.

Effect of butyrate and/or CO$_2$/HCO$_3^-$ on [Mg$^{2+}$]$_i$ and a comparison of Mg$^{2+}$ uptake in high-NaCl and high-KCl medium. As in our previous study (37), the [Mg$^{2+}$]$_i$ of REC incubated in a high-NaCl medium increases after exposure to butyrate and/or CO$_2$/HCO$_3^-$. This
In the high-NaCl medium, viz., which are known inhibitors of cation-Cl sulfamoyl-benzoic acid-type diuretics (loop diuretics), Mg\textsuperscript{2+} transport systems for Mg\textsuperscript{2+} are strongly opposed by the enteric transport of Mg\textsuperscript{2+} by a cotransporter (37). The results obtained in the high-NaCl medium, viz., 1) that butyrate alone is not able to stimulate Mg\textsuperscript{2+} uptake and 2) that the rate of Mg\textsuperscript{2+} uptake is the same in HCO\textsubscript{3}⁻-buffered media and HCO\textsubscript{3}⁻-buffered media with additional butyrate, have led us to presume that the Mg\textsuperscript{2+} influx is coupled to HCO\textsubscript{3}⁻ uptake via a Mg\textsuperscript{2+}-2HCO\textsubscript{3}⁻ cotransport. Such a transport system has been shown by Günther et al. (14) in Yoshida ascites tumor cells. However, the existence of a Mg\textsuperscript{2+}-2HCO\textsubscript{3}⁻ cotransport is strongly opposed by the finding that the stimulating effect of HCO\textsubscript{3}⁻ is completely abolished after reduction of the [Cl\textsuperscript{−}]\textsubscript{i}. In the experiments performed by Günther et al. (14), even the total removal of Cl\textsuperscript{−} from the extracellular solution had no negative influence on the Mg\textsuperscript{2+} uptake as long as HCO\textsubscript{3}⁻ was present. Another result mitigating against a Mg\textsuperscript{2+}-2HCO\textsubscript{3}⁻ cotransport is the significant reduction of Mg\textsuperscript{2+} influx in the HCO\textsubscript{3}⁻-buffered high-KCl medium.

**Effect of [Cl\textsuperscript{−}]\textsubscript{i}**. On the other hand, the [Cl\textsuperscript{−}]\textsubscript{i}, directly influences the [Mg\textsuperscript{2+}]\textsubscript{i} and the Mg\textsuperscript{2+} influx rate. Pre-incubation of REC in a Cl\textsuperscript{−}-reduced medium (36 mM) causes a decrease in the [Mg\textsuperscript{2+}]\textsubscript{i} and a complete inhibition of the CO\textsubscript{2}/HCO\textsubscript{3}⁻-stimulated Mg\textsuperscript{2+} uptake. We interpret this data as reflecting that the K\textsuperscript{−}-insensitive Mg\textsuperscript{2+} influx in REC is mediated by a cotransport of Mg\textsuperscript{2+} with Cl\textsuperscript{−}. This idea is also supported by the sensitivity of [Mg\textsuperscript{2+}]\textsubscript{i}, and the Mg\textsuperscript{2+} uptake to the sulfamoyl-benzoic acid-type diuretics (loop diuretics), which are known inhibitors of cation-Cl cotransporters (26, 29). Bumetanide and furosemide (100 μM each) reduced the rate of Mg\textsuperscript{2+} influx by 64 and 40%, respectively, showing that bumetanide is the more potent blocker. This relative sensitivity to loop diuretics (bumetanide > furosemide) and the insensitivity to the thiazide-type agent chlorothiazide is typical for transport proteins belonging to the sodium-(potassium)-chloride cotransporter family (10, 32). It is important to note, however, that potassium is not universally required and that a number of studies have suggested the existence of bumetanide-sensitive, thiazide-insensitive Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporters (40). Anion-dependent transport systems for Mg\textsuperscript{2+} influx and Mg\textsuperscript{2+} efflux have been described previously (13, 19, 28). The existence of an Mg\textsuperscript{2+}–Cl\textsuperscript{−} cotransporter in rumen epithelium is in accordance with results from controlled feeding trials with sheep showing a positive effect of Cl\textsuperscript{−} on Mg\textsuperscript{2+} absorption (34). Furthermore, in vitro experiments with isolated sheep rumen epithelia have revealed that the cellular component of Mg\textsuperscript{2+} absorption is strongly reduced by the removal of apical Cl\textsuperscript{−}. However, the effect of Cl\textsuperscript{−} withdrawal had been taken to reflect the coupling of an Mg\textsuperscript{2+}/H\textsuperscript{+} exchanger and a Cl\textsuperscript{−}/HCO\textsubscript{3}⁻ exchanger (23). Because we have found no evidence for such a double ion exchange mechanism, we have tested an alternative hypothesis.

For some epithelia, it has been shown that a proton-motive force generated by electrogenic H\textsuperscript{+}–ATPases in the apical membrane is utilized as a driving force for numerous transport processes (16, 18). Because the membrane potential has been shown to be the main driving force for Mg\textsuperscript{2+} uptake into REC (22, 35) and because of the positive relationship between intracellular H\textsuperscript{+} availability and transepithelial Mg\textsuperscript{2+} transport (23), we have tested whether a H\textsuperscript{+} pump is involved in Mg\textsuperscript{2+} uptake. For this purpose, we have used baflomycin A\textsubscript{1} and folicomycin, which are known to be potent inhibitors of vacuolar ATPases (V-ATPases) (3, 6). REC [Mg\textsuperscript{2+}]\textsubscript{i} and Mg\textsuperscript{2+} influx are reduced by 36–38% after preincubation with baflomycin A\textsubscript{1} or folicomycin, respectively, supporting the idea that a vacuolar H\textsuperscript{+}-ATPase energizes Mg\textsuperscript{2+} influx by generating an inside-negative membrane potential. The protons needed for this process are produced in cytoplasm from CO\textsubscript{2} and water by the enzyme carbonic anhydrase. The presence of the latter in REC has been reported repeatedly (1, 2). For high concentrations of furosemide and related compounds, an interference with carbonic anhydrase has been shown (29). Thus some of the negative effects of furosemide on Mg\textsuperscript{2+} uptake may result from an inhibition of the enzyme. HCO\textsubscript{3}⁻, which is also formed from this reaction, is secreted through the cell membrane in exchange for Cl\textsuperscript{−} and/or SCFA\textsuperscript{−}, but it is not clear whether Cl\textsuperscript{−} and SCFA\textsuperscript{−} compete for extracellular binding sites at a common anion-exchange mechanism or whether there are separate Cl\textsuperscript{−}/HCO\textsubscript{3}⁻ and SCFA /HCO\textsubscript{3}⁻ exchangers (8, 21, 39). However, a reduction of the apical [Cl\textsuperscript{−}] or a Cl\textsuperscript{−} withdrawal from the luminal site of the epithelium leads to an elevation of the cellular SCFA transport (21, 39). This makes it likely that butyrate anions effect Mg\textsuperscript{2+} transport indirectly by stimulating HCO\textsubscript{3}⁻ secretion and that they therefore increase intracellular H\textsuperscript{+} availability to the H\textsuperscript{+} pump. In this way, butyrate can partly substitute for extracellular Cl\textsuperscript{−} in the CO\textsubscript{2}/HCO\textsubscript{3}⁻-buffered solution. Because a great proportion (~95%) under in vivo conditions) of absorbed butyrate is metabolized to CO\textsubscript{2} in REC (38), the positive effect of SCFA also results from its delivery substrate to the carbonic anhydrase reaction. Oxidative metabolism of SCFA is an important energy source for the epithelial cells themselves (4), and the reversible disassembly of V-ATPase into its V\textsubscript{0} and V\textsubscript{1} subunits acts as a type of regulation of its activity in response to a drop in energy supply (25). The coupling of REC energy metabolism, H\textsuperscript{+}-ATPase activity, and Mg\textsuperscript{2+} uptake provides an explanation of the positive effect of easily fermentable carbohydrates on Mg\textsuperscript{2+} absorption (7, 11). CO\textsubscript{2} and SCFA also stimulate Mg\textsuperscript{2+} absorption from the large intestine (colon, cecum) of various species (5, 31, 33), but knowledge of the underlying mechanisms is limited. Holtug (17) has
found evidence for SCFA–dependent active proton secretion in the colon of the hen. The implication of such a mechanism for Mg2+ transport in the large intestine of mammals remains to be shown.

In conclusion, our results are in agreement with the existence of an Mg2+-anion cotransport, most probably an Mg2+-Cl– cotransport, in the cell membrane of REC. At this time, we cannot exclude that other ions (HCO3–) can substitute for Cl– under certain conditions. Furthermore, it seems likely from our experiments with inhibitors of the vacuolar H+–ATPase (bafilomycin A1, fomiycin) that this Mg2+ uptake is energized by electrogenic proton pumps, which initially generate a transmembrane inside-negative voltage. The parallel influx of Mg2+ and Cl– dissipates this voltage, which is an explanation for results showing the existence of a PD- and K+ voltage, which is an explanation for results showing a Mg2+ efflux of Mg2+ across rumen epithelium. The parallel influx of Mg2+ and Cl– dissipates this voltage, which is an explanation for results showing the existence of a PD- and K+-dependent component and a so-called “PD- or K+-insensitive” component of ruminal Mg2+ transport (22, 35). The positive effect of ruminal fermentation products, such as SCFA/SCFA and CO2, on Mg2+ transport seems to be an indirect one and can be explained by their influence on the activity of a vacuolar H+-ATPase, thereby increasing the driving force for the uptake of Mg2+.

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