Chloride, Gluconate, Sulfate, and Short-Chain Fatty Acids Affect Calcium Flux Rates Across the Sheep Forestomach Epithelium

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ABSTRACT

In ruminants, more than 50% of overall gastrointestinal Ca absorption can occur preintestinally, and the anions of orally applied Ca salts are thought to play an important role in stimulating ruminal Ca absorption. This assumption is based mainly on ion-exchange studies that have used gluconate as the control anion, which may bind Ca^{2+} ions and interfere with treatment effects. In the present study, we investigated the distinct effects of different anions on Ca absorption across the sheep rumen and on the concentration of free Ca^{2+} ions ($[\text{Ca}^{2+}]_{\text{ion}}$). We showed that gluconate, sulfate, and short-chain fatty acids (SCFA) remarkably reduced $[\text{Ca}^{2+}]_{\text{ion}}$ in buffer solutions. Nevertheless, increasing the Cl or SCFA concentration by 60 mM stimulated net ruminal Ca absorption 5- to 7-fold, but these effects could be antagonized by gluconate. Therefore, ion-exchange experiments must be (re)evaluated very carefully, because changes in $[\text{Ca}^{2+}]_{\text{ion}}$ in the presence of gluconate, sulfate, or SCFA not only might entail an underestimation of Ca flux rates, but also might have effects on other cellular pathways that are Ca^{2+} dependent. Concerning the optimal Ca supply for dairy cows, the present study suggests that CaCl_2 formulations and Ca salts of the SCFA stimulate Ca absorption across the rumen wall and are beneficial in preventing or correcting a Ca deficiency.

Key words: calcium absorption, chloride, rumen, short-chain fatty acid

INTRODUCTION

In ruminants, gastrointestinal Ca absorption can occur in the small (and large) intestine, as is known from Ca absorption in monogastric animals. In addition, a remarkable proportion of Ca is absorbed across the epithelium of the rumen (Grace et al., 1974; Rayssiguier and Poncet, 1980), which is the biggest forestomach. In vivo studies with the isolated rumen of sheep have

shown a net absorption of 5 μmol of Ca per L and min (Wadhwa and Care, 2000), which can be extrapolated to a ruminal Ca absorption of 1.7 g/d, assuming a volume of 6 L of rumen fluid. Using the same method, Wagner (1998) measured a ruminal Ca absorption of between 77 and 157 $\mu\text{mol/h}$ per $\text{kg}^{0.75}$ of BW. Because the BW of sheep in Wagner's (1998) study ranged from 48 to 101 kg, this rate can be extrapolated to a ruminal Ca absorption of between 1.3 and 4.8 g/d.

Feeding experiments have shown that the preintestinal localization of Ca absorption gains importance if Ca intake increases (Schröder, 1996; Khorasani et al., 1997). Sheep with a Ca intake of 2.7 g/d showed a preintestinal Ca secretion of 0.8 g/d and an intestinal Ca absorption of 1.6 g/d (Wylie et al., 1985), whereas sheep with a Ca intake of between 4.8 and 13.6 g/d showed a preintestinal Ca absorption of between 0.7 and 4.3 g/d and a Ca secretion in the small intestine of between 1.2 and 2.4 g/d, which was partly compensated for by Ca absorption in the large intestine (Grace et al., 1974). Comparable observations have been made in cows. Lactating cows with a Ca intake of between 115 and 231 g/d showed a preintestinal Ca movement ranging from a small secretion of -0.9 g/d at a low Ca intake to an absorption of 49.8 g/d at a high Ca intake (Khorasani et al., 1997).

The addition of Ca salts to the diets of cows is a frequent strategy at the onset of lactation, because dairy cows lose considerable amounts of calcium to milk production. Compared with an immediately available Ca pool of 3 to 4 g in the circulating blood of a cow (calculated on the basis of ~ 2.25 mM Ca and ~ 65 mL of blood/kg of BW), the Ca drain with milk can amount to 50 g/d (assuming a content of 1.2 g of Ca/L of milk). To prevent or correct a Ca imbalance in lactating cows, Ca salts are added to their diet (e.g., Ca-chloride, Ca-propionate). After ingesting the diet, these salts dissociate in the rumen fluid; therefore, the respective anions may influence Ca transport rates across the rumen epithelium.

The mechanisms of ruminal Ca absorption have been studied in small ruminants, where different groups have shown the absorption of Ca across the rumen of sheep to be stimulated by short-chain fatty acids

Received June 9, 2006.

Accepted October 2, 2006.

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(SCFA; Schröder et al., 1997, 1999; Wadhwa and Care, 2000; Uppal et al., 2003) and by feeding concentrate (Uppal et al., 2003). Further, preliminary studies have suggested a stimulatory effect on ruminal Ca absorption by chloride anions (Leonhard-Marek et al., 2000). However, all those experiments were conducted as anion-exchange in vitro studies in which the anions investigated were replaced by gluconate in the control solutions. This may be a disadvantage, because gluconate is capable of binding Ca^{2+} ions to some extent (Skibsted and Kilde, 1972; Christoffersen and Skibsted, 1975; Kenyon and Gibbons, 1977). This knowledge has not been considered in most measurements of the Ca flux rates.

We therefore studied the distinct effects of chloride, SCFA, gluconate, and sulfate on Ca absorption across isolated epithelia from sheep rumen and measured the effects of these anions on ionized Ca^{2+} concentrations ($[\text{Ca}^{2+}]_{\text{ion}}$) in the bathing solutions.

MATERIALS AND METHODS

Tissues

The protocol for the animal treatment was approved and its conduct supervised by the animal protection officer of the Department of Physiology, School of Veterinary Medicine. Adult male and female German black-headed mutton sheep (19 animals, 7 to 8 mo of age) were killed by captive bolt stunning followed by exsanguination from the carotid arteries. Within 5 min after slaughter, a piece of about 15×20 cm of the ventral rumen wall was taken and immediately immersed in a buffer solution containing SCFA (solution A; Table 1) at 38°C , where the mucosa was stripped from the underlying muscle layers and the serosa (Leonhard-Marek et al., 1998). The tissue was then cut into smaller pieces of about 2×2 cm.

Solutions

Because SCFA are the major anions on the luminal side under in vivo conditions, we started the first experiments with a physiological SCFA gradient across the rumen wall. Epithelia were bathed with buffer solutions containing a mixture of 60 mM SCFA on the mucosal side (Table 1, buffer A). These SCFA anions were replaced by 60 mM gluconate in the solution bathing the serosal side of the epithelia (buffer C). Both solutions contained 68 mM Cl. In these first experiments, Cl was replaced by gluconate to obtain a low-Cl solution (buffer B).

In the second series of experiments, we (re)evaluated the effects of different anions on Ca flux rates, starting from a control buffer that contained mannitol (and no

gluconate, buffer D; Table 1). To obtain different anion solutions, we replaced mannitol by Tris-OH and the respective acid (hydrochloric acid, sulfuric acid, or SCFA, buffers E to G; Table 1).

The compositions of the different solutions are presented in Table 1. All solutions had a pH of 7.4 when gassed with 95% O_2 and 5% CO_2 at 38°C and an osmolarity of 300 mosmol/L. All chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany) or from Sigma-Aldrich (Deisenhofen, Germany).

Electrical Measurements

Mucosal tissues were mounted between the two halves of Ussing incubation chambers (Department of Physiology, School of Veterinary Medicine, Hannover, Germany) with an exposed area of 1.13 cm^2 . Damage to the tissue edges was minimized by placing rings of silicon rubber on both sides of the tissues. Incubation chambers were connected to circulation reservoirs (glassblower shop, Hannover Medical School, Hannover, Germany) containing 10 mL of buffer solution on each side. The solutions were maintained at 38°C by a water jacket and were continuously stirred by use of a gas lift system that supplied 95% O_2 and 5% CO_2 . The Ussing chambers were also connected to a computer-controlled voltage clamp device (Mussler Scientific Instruments, Aachen, Germany). Transepithelial potential differences (V_t) were measured through buffer solution agar bridges and calomel electrodes with reference to the mucosal solution. Transepithelial tissue conductances (G_t) were determined from the changes in V_t induced by bipolar current pulses of $100 \mu\text{A}/\text{cm}^2$ of 500 ms duration. The currents were passed through buffer solution agar bridges connected to Ag-AgCl electrodes in 3 M KCl. In each setup, fluid resistances and junction potentials were measured before mounting the mucosal tissues and were corrected for during the experiments. The experiments were performed under short-circuit conditions, unless otherwise stated.

Ca Flux Rates

Unidirectional flux rates of Ca were measured using ^{45}Ca as a tracer, which was added as $^{45}\text{CaCl}_2$ (specific activity $>370 \text{ GBq/g}$; PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany) in each chamber to the mucosal or the serosal side of the epithelia. After an equilibration period of 20 min, 10 to 12 buffer samples of 500 μL were taken at 30-min intervals and replaced by aliquots of the same unlabeled solution. Radioactivity was measured in a conventional liquid scintillation counter (Tricarb Packard, Dreieich, Germany). Flux rates were calculated from the rate of tracer appearance

Table 1. Composition of the buffer solutions¹

Composition	Experiment 1		
	Buffer A, high Cl/SCFA ²	Buffer B, low Cl/ SCFA gluconate ²	Buffer C, high Cl/ SCFA-free gluconate ³
NaCl	57	—	57
KCl	5	5	5
HCl	0.8	—	0.4
CaCl ₂	1.2	1.2	1.2
MgCl ₂	1.2	1.2	1.2
NaHCO ₃	21	21	21
Na ₂ HPO ₄	1.2	1.2	1.4
NaH ₂ PO ₄	1.6	1.6	1.4
Glucose	—	—	5
Na-acetate	36	36	—
Na-propionate	15	15	—
Na- <i>n</i> -butyrate	9	9	—
Na-gluconate	1.2	58.2	61
Mannitol	18	23	20

Composition	Experiment 2			
	Buffer D, control/mannitol ^{2,3}	Buffer E, high Cl ²	Buffer F, high SO ₄ ²	Buffer G, high SCFA ²
KCl	5	5	5	5
CaCl ₂	1.2	1.2	1.2	1.2
MgCl ₂	1.2	1.2	1.2	1.2
NaHCO ₃	21	21	21	21
Na ₂ HPO ₄	1.2	1.2	1.2	1.2
NaH ₂ PO ₄	1.6	1.6	1.6	1.6
Glucose	10	10	10	10
HCl	1	60.2	0.2	0.2
H ₂ SO ₄	—	—	30	—
Acetic acid	—	—	—	36
Propionic acid	—	—	—	15
<i>n</i> -Butyric acid	—	—	—	9
Tris-OH	—	65.9	116	70
Mannitol	217.5	110	90	100

¹Concentrations are given in mM. SCFA = short-chain fatty acids.²Solution used on the mucosal side of the epithelia.³Solution used on the serosal side of the epithelia.

on the other side of the epithelia. In the absence of any manipulation, flux rates were stable from the second to the ninth flux period in the epithelia used for the experiments under short-circuit conditions, and from the third to the eleventh flux period in the epithelia used for the voltage clamp experiments. On the basis of steady-state values, a mean flux rate was calculated for each epithelial sheet and each condition. The effects of different anions on Ca flux rates were tested with adjacent pieces of epithelia from the same sheep.

To study the effect of different transepithelial potential differences on Ca flux rates, we incubated 2 epithelia for each buffer solution and each flux direction from each sheep. One of these tissues was subsequently clamped to -25, +25, and 0 mV, and the other tissue was clamped to +25, -25, and 0 mV. To exclude an influence of the sequence of the applied potential differences, the steady-state fluxes measured at the respective V_t were averaged between the 2 tissues. Unidirec-

tional fluxes measured at different potential differences were divided into their electrogenic and electroneutral components according to a mathematical model described by Frizzell and Schultz (1972): $\mathbf{J} = \mathbf{J}_d \cdot \xi^{-0.5} + \mathbf{J}_m$, and $\xi = e^{(zFV/RT)}$, where $\mathbf{J}$ is the total unidirectional flux of an ion, $\mathbf{J}_d \cdot \xi^{-0.5}$ is its diffusible (electrogenic) part, and $\mathbf{J}_m$ is its electroneutral (voltage-independent) proportion. V gives the potential difference across the epithelium in flux direction, z is the valence of the ion, F is the Faraday constant, R is the gas constant, and T is the absolute temperature.

Calcium flux rates were measured 1) in the presence of gluconate or chloride (corresponding to the classical ion-exchange experiments) and at different transepithelial potential differences (allowing for the calculation of electrogenic and electroneutral flux components); 2) in the presence of gluconate and chloride (to follow up on the distinct effects of each anion on the same tissues; see below); and 3) in the absence of gluco-

Figure 1. Calcium flux rates at different Cl concentrations on the mucosal side. JCa_{ms} = Ca flux rate from the mucosal to the serosal side ($n = 12$); JCa_{sm} = Ca flux rate from the serosal to the mucosal side ($n = 6$). Low Cl (10 mM Cl, 58 mM gluconate; buffer B), high Cl (68 mM Cl; buffer A); * $P < 0.05$ vs. high Cl.

nate (allowing us to monitor the specific effect of an anion in relation to a mannitol control).

For the second purpose, we added gluconate to a high-Cl solution or Cl to a high-gluconate solution. The addition of these salts increased the osmolarity by about 100 mosmol/L on the luminal side. This increase is in the range of changes that can occur after food intake (Warner and Stacy, 1965; von Engelhardt, 1969) and induces changes in transepithelial conductance that can be measured in *in vitro* studies. These increases in G_t have been attributed to an increase in passive paracellular movements (Schweigel et al., 2005). Apart from these osmolarity-induced increases in G_t , the transepithelial conductance, which was taken as a measure of tissue viability, remained stable for the duration of the experiments.

Ionized Ca^{2+}

The concentrations of free Ca^{2+} ions in the solutions were measured with a blood gas and electrolyte analyzer (Bayer Diagnostics, Fenwal, Germany) equipped with an ion-selective Ca^{2+} electrode. Samples were analyzed at 37°C and corrected to a pH of 7.4. The ionized Ca^{2+} concentration was measured 1) in gluconate and Cl solutions at different times, in parallel to the protocol that had been used for the flux experiments; 2) after addition of gluconate or chloride; and 3) in mannitol, sulfate, and SCFA solutions.

Statistics

Results are given as means ± standard error of the means. Unless otherwise stated, n represents the num-

ber of tissues. Wherever possible, 2 pieces of tissues were incubated per animal and per experimental condition. Epithelial preparations that showed large unspecific increases in transepithelial conductance were excluded from the experiments. Most experiments were conducted with tissues from a group of 6 animals; in 2 instances, 9 and 4 animals were available. Statistical significance was evaluated using ANOVA (one-way) if 3 or more groups had to be compared. For the comparison of 2 conditions, we used Student's *t*-test, paired or unpaired as appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

Ca Flux Rates in the Presence of Gluconate or Chloride

In the first experiments with a physiological SCFA gradient across the rumen wall, epithelia were bathed with buffer solutions containing a mixture of 60 mM SCFA on the mucosal side (buffer A; Table 1). These SCFA anions were replaced by gluconate on the serosal side of the epithelia (buffer C). Both solutions contained 68 mM Cl. Under this high-Cl condition, we observed a Ca flux rate from the mucosal to the serosal side (JCa_{ms}) of 20.3 ± 1.4 nmol cm⁻² h⁻¹ ($n = 12$; Figure 1) and a Ca flux rate from the serosal to the mucosal compartment (JCa_{sm}) of 2.3 ± 0.4 nmol cm⁻² h⁻¹ ($n = 6$), resulting in a significant net Ca absorption ($JCa_{net} = JCa_{ms} - JCa_{sm}$) of 18.0 ± 1.1 nmol cm⁻² h⁻¹ ($n = 6$) across the rumen epithelium.

Adjacent epithelia from the same animals were incubated with a reduced Cl concentration on the mucosal side (10 mM Cl and 58 mM gluconate; buffer B). This altered luminal condition had no effect on JCa_{sm} (2.7 ± 0.5 nmol cm⁻² h⁻¹, $n = 6$; Figure 1), but JCa_{ms} was significantly reduced to 13.4 ± 0.7 nmol cm⁻² h⁻¹ ($n = 12$, $P < 0.001$ vs. high Cl). The JCa_{net} was reduced to 10.7 ± 1.1 nmol cm⁻² h⁻¹ ($n = 6$).

The results (Figure 1) showed a significant effect of Cl (or gluconate) on JCa_{ms} under short-circuit conditions (at zero electrochemical potential). Subsequently, we measured JCa_{ms} and JCa_{sm} at +25, 0, and -25 mV of V_t . The analysis of these Ca flux rates at varying V_t (according to Frizzell and Schultz, 1972; see the Materials and Methods section) and linear regressions of the Ca fluxes on the electric driving force ($\xi^{-0.5}$) resulted in the following equations ($n = 27$ observations in 9 sheep):

High-Cl

$$JCa_{ms} = 10.4 (\pm 2.5) + 9.6 (\pm 1.6) \xi^{-0.5}$$

$$(r^2 = 0.604, P < 0.001),$$

$$JCa_{sm} = 2.1 (\pm 0.6) + 1.1 (\pm 0.4) \xi^{-0.5}$$

$$(r^2 = 0.255, P < 0.01),$$

Low-Cl

$$JCa_{ms} = 6.8 (\pm 1.8) + 6.4 (\pm 1.1) \xi^{-0.5}$$

$$(r^2 = 0.576, P < 0.001),$$

$$JCa_{sm} = 3.5 (\pm 0.6) + 1.1 (\pm 0.4) \xi^{-0.5}$$

$$(r^2 = 0.225, P < 0.05).$$

A comparison of the Cl effects on the basis of the number of animals ($n = 9$) showed that Cl increased both the electrogenic and the electroneutral component of JCa_{ms} ($P < 0.01$). Chloride also had a small but significant effect on the electroneutral component of JCa_{sm} ($P < 0.05$), whereas the electrogenic component of JCa_{sm} remained unaffected.

Ca Flux Rates in the Presence of Gluconate and Chloride

This first study, corresponding to the classical ion-exchange experiments, did not allow us to discriminate between a stimulatory effect of Cl or a blocking effect of gluconate. We therefore simulated an additional salt intake after the fifth flux period and added 58 mM NaCl to the luminal side of the epithelia bathed in the low-Cl (and high-gluconate) buffer. At the same time, adjacent epithelia that were incubated in a high-Cl (and low-gluconate) buffer received an addition of 58 mM Na-gluconate to the luminal side (Figure 2). Both groups of epithelia thus faced the same composition of buffer solution during the final flux rates.

Addition of these salts increased osmolarity by about 100 mosmol/L on the luminal side and induced an increase in G_t from 2.0 ± 0.2 to 3.1 ± 0.3 mS cm⁻² ($n = 6$, $P < 0.001$) after the addition of NaCl, and from 2.2 ± 0.2 to 3.2 ± 0.2 mS cm⁻² ($n = 6$, $P < 0.001$) after the addition of Na-gluconate. This increase in G_t was accompanied by a parallel increase in JCa_{sm} , from 2.7 ± 0.5 to 6.1 ± 1.2 nmol cm⁻² h⁻¹ after the addition of NaCl and from 2.3 ± 0.4 to 6.6 ± 1.2 nmol cm⁻² h⁻¹ after Na-gluconate (for both groups $n = 6$, $P < 0.01$), in line with an increased paracellular conductance.

Despite the changes in the paracellular pathway, the addition of Cl ions to the low-Cl, high-gluconate solution had no effect on JCa_{ms} (14.0 ± 1.1 nmol cm⁻² h⁻¹ before vs. 13.1 ± 2.4 nmol cm⁻² h⁻¹ after addition of chloride, $n = 6$). In contrast, the addition of gluconate to the high-Cl, low-gluconate solution reduced JCa_{ms} from 18.9 ± 1.8 nmol cm⁻² h⁻¹ (before addition of gluconate) to 11.5

Figure 2. Calcium flux rates from the mucosal to the serosal side (JCa_{ms}) at different Cl and gluconate concentrations on the mucosal side. For the first column of each pair (gray bars), we calculated a mean flux rate for each epithelium on the basis of steady-state Ca fluxes from the second to the fifth flux period. The second column of each pair (black bars) represents the eighth flux period (between 50 and 80 min after addition of Cl or gluconate, $n = 6$); ** $P < 0.01$ vs. 68 mM Cl, gluconate free.

± 0.5 nmol cm⁻² h⁻¹ ($n = 6$, $P < 0.01$; Figure 2). These data showed that, in a standard ion-exchange experiment, the higher Ca flux rates in a Cl buffer were not due to a stimulating effect of Cl, but could be attributed to a blocking effect of gluconate in the control solution.

Ionized Ca²⁺ Concentration in Gluconate and Cl Solutions

The extent of JCa_{ms} reduction attributable to gluconate was rather astonishing. We therefore expanded the study by measuring the concentration of free Ca²⁺ ions in all buffer solutions (Figure 3). All buffer solutions had a total Ca concentration of 1.2 mM. Of these we found 0.8 ± 0.02 mM $[Ca^{2+}]_{ion}$ in the high-Cl (SCFA) solution (buffer A; $n = 4$ freshly prepared solutions), but only 0.2 ± 0.0 mM $[Ca^{2+}]_{ion}$ in the low-Cl solution (high-gluconate and SCFA, buffer B; $n = 3$ solutions). The buffer solution on the serosal side (high-Cl, high-gluconate, no SCFA, buffer C) showed a $[Ca^{2+}]_{ion}$ concentration of 0.3 ± 0.03 mM ($n = 4$ solutions). These concentrations of $[Ca^{2+}]_{ion}$ did not change between measurements carried out in the buffer reservoir in the Ussing chambers 5 min before the placement of the epithelia and 300 min after incubation of the epithelia (Figure 3), indicating that the tissues themselves had no effect on the concentration of $[Ca^{2+}]_{ion}$. We then repeated the Ca flux protocol and added Na-gluconate or NaCl (58 mM each) to the buffer solutions on the mucosal side. Gluconate immediately decreased the concentration of free Ca²⁺ ions to 0.3 ± 0.01 mM ($P < 0.001$, $n = 5$; Figure 3), in parallel with the reduction of the Ca flux rates

Figure 3. Concentration of free Ca ions ($[Ca^{2+}]_{ion}$) in different buffer solutions during the course of a flux experiment. Buffer solutions are the same as in the flux experiment presented in Figure 2. High Cl (68 mM Cl; buffer A), low Cl (10 mM Cl, 58 mM gluconate; buffer B), + gluconate or + Cl (addition of 58 mM Na-gluconate or 58 mM NaCl, respectively, to the mucosal side at $t = 190$ min, shown by the arrow; $n = 4$ to 5). Means $\pm$ SEM (some error bars are smaller than the symbols).

shown above (JCa_{ms} ; Figure 2). Chloride, which had no effect on JCa_{ms} (Figure 2), had also almost no effect on the concentration of free Ca^{2+} ions (0.27 ± 0.01 mM before and 0.29 ± 0.01 mM after addition of 58 mM NaCl, $P = 0.07$, $n = 5$; Figure 3).

The results of our first experiment thus showed us a huge effect of gluconate on Ca flux rates, which called into question the anion effects of Cl and SCFA reported in the literature (using gluconate as the control anion).

Effect of Anions on Ca Fluxes (Without Gluconate)

In a second series, we (re)evaluated the effects of different anions on Ca flux rates, starting from a control buffer that contained mannitol (and no gluconate; buffer D in Table 1). To obtain different anion solutions, we replaced mannitol by Tris-OH and the respective acid (hydrochloric acid, sulfuric acid, or SCFA; buffers E to G in Table 1 and Figure 4).

Under control conditions, JCa_{ms} amounted to 8.3 ± 0.4 nmol $cm^{-2} h^{-1}$ and JCa_{sm} to 6.2 ± 0.4 nmol $cm^{-2} h^{-1}$. Changing the luminal condition to different dominant anions had no effect on JCa_{sm} . Luminal sulfate also had no effect on JCa_{ms} . However, increasing the luminal concentration of Cl and SCFA significantly increased JCa_{ms} , which resulted in significant net absorption rates for Ca under these conditions (Figure 4).

Ionized Ca^{2+} Concentration in Cl, SCFA, and Sulfate Solutions

To compare the observed Ca flux rates to the concentration of free Ca^{2+} ions, we again measured the concen-

Figure 4. Calcium flux rates measured in the presence of different dominant anions on the mucosal side. JCa_{ms} = Ca flux rate from the mucosal to the serosal side; JCa_{sm} = Ca flux from the serosal to the mucosal side; JCa_{net} = net Ca absorption; SCFA = short-chain fatty acids. Buffers D to G are described in Table 1; $n = 4$ to 8 epithelia from 4 sheep. * $P < 0.05$, significant net absorption for JCa_{ms} vs. JCa_{sm} .

tration of ionized Ca^{2+} in these solutions. As in the first series, the total Ca concentration amounted to 1.2 mM in all buffer solutions. These measurements showed that not only gluconate, but also sulfate and SCFA could reduce the concentration of ionized Ca^{2+} (Table 2), which was not always correlated with a reduction in the Ca flux rate.

DISCUSSION

Effects of Cl on Ruminal Ca Absorption

Because $CaCl_2$ is frequently used as an oral therapeutic in cases of suspected or diagnosed hypocalcemia in ruminants, we wanted to know whether Cl ions might influence Ca absorption across the rumen wall. Although a high Ca intake has been shown to result in preintestinal Ca absorption in sheep and cows, the mechanisms involved in ruminal Ca transport have been studied more intensively in sheep. To allow for a better comparison with data from the literature, we conducted this study with epithelia from sheep rumen. To study the isolated effects of Cl ions, we exchanged mannitol for Tris-Cl without increasing the free Ca^{2+} concentration (Table 1) and found that Cl is indeed able to stimulate JCa_{ms} and net Ca absorption from the rumen (Figure 4).

Table 2. Effect of different selected anions on the concentration of free Ca^{2+} ions and Ca^{2+} flux rates¹

Anion	Buffer D, control	Buffer E, high Cl	Buffer F, high SO_4	Buffer G, high SCFA
Cl^- , mM	10	70	10	10
SCFA ⁻ , mM	0	0	0	60
SO_4^{2-} , mM	0	0	30	0
$[\text{Ca}^{2+}]_{\text{ion}}$, mM	1.11 ± 0.01	$1.02 \pm 0.00^*$	$0.36 \pm 0.01^*$	$0.77 \pm 0.01^*$
$J\text{Ca}_{\text{ms}}$, nmol $\text{cm}^{-2} \text{h}^{-1}$	8.3 ± 0.4	14.5 ± 1.4	6.0 ± 0.5	19.2 ± 3.0

¹Other unnamed anions were kept at a constant level (see Table 1). $[\text{Ca}^{2+}]_{\text{ion}}$ = concentration of free Ca^{2+} ions ($n = 4$), $^*P < 0.05$ vs. control; $J\text{Ca}_{\text{ms}}$ = Ca^{2+} flux rate from the mucosal to the serosal side ($n = 6$ to 8 epithelia from 4 sheep).

Calcium absorption across the sheep rumen epithelium is at least partly active (Höller et al., 1988; Schröder et al., 1997) and involves electrogenic and electroneutral components (Höller et al., 1988; Wadhwa and Care, 2000), which was confirmed here. The electrogenic component of $J\text{Ca}_{\text{ms}}$ cannot be explained by mere paracellular transport, because it clearly differed from the respective component of $J\text{Ca}_{\text{sm}}$. The majority of potential-dependent $J\text{Ca}_{\text{ms}}$ has therefore to be considered as an electrogenic transport through the cells (Figure 5). Both components of $J\text{Ca}_{\text{ms}}$, electrogenic and electroneutral, were stimulated by mucosal Cl in the present study.

The current concept of Cl absorption across the rumen epithelium assumes an electroneutral Cl uptake across the apical membrane via $\text{Cl}^-/\text{HCO}_3^-$ exchange

(Chien and Stevens, 1972; Martens et al., 1991). Candidates for this exchange are anion exchanger 2, the intestinal anion exchanger DRA (down-regulated in adenoma), and the putative anion transporter 1, which have been shown on the mRNA level in rumen epithelia and cultured rumen epithelial cells from sheep (Bilk et al., 2005). Recent patch clamp experiments with isolated rumen epithelial cells showed that rumen epithelial cells also express a Cl^- conductance (Leonhard-Marek et al., 2005). In microelectrode experiments, where we impaled rumen epithelium via the apical membrane, we could show that this Cl^- conductance must be localized in the basolateral membranes of rumen epithelial cells (Leonhard-Marek et al., 2006), where it might contribute to the transepithelial transport of Cl, as suggested for basolateral-localized Cl

Figure 5. Schematic overview of transport processes across the rumen epithelium that could contribute to the stimulation of ruminal Ca absorption. SCFA⁻ = short-chain fatty acid anion; H-SCFA = protonated short-chain fatty acid.

channels in the distal colon of guinea pigs (ClC-2; Catalan et al., 2002). In line with the electroneutral uptake of Cl via luminal anion exchangers and electrogenic extrusion of Cl across the basolateral membrane, an acute increase in luminal Cl concentration depolarized the apical membrane of the rumen epithelium (Leonhard-Marek et al., 2006). Therefore, an electrogenic stimulation of Ca^{2+} transport via a Cl-induced alteration of electric driving forces seems rather unlikely.

Luminal Cl is, however, able to increase the pH at the epithelial surface via a stimulation of apical localized $\text{Cl}^-/\text{HCO}_3^-$ or Cl^-/OH^- transporters (Figure 5; Leonhard-Marek et al., 2006). In vivo studies with the temporarily isolated rumen have shown that an increase in luminal pH can in turn increase ruminal Ca absorption (Wagner, 1998). The structures of the proteins involved in ruminal Ca transport are not yet known, but we have recently shown that the apical membrane of rumen epithelia from sheep and goats exhibits a nonselective cation conductance, which allows for a current of monovalent cations in the absence of divalent cations and is regulated by extracellular Ca^{2+} and Mg^{2+} as well as by intracellular Mg^{2+} ions (Leonhard-Marek, 2002; Leonhard-Marek et al., 2005). This phenomenon, the passage of monovalents in the absence of divalent cations, is a feature common to many Ca^{2+} channels (Sather and McCleskey, 2003; Hoenderop et al., 2005). In the rumen, the Ca^{2+} channel agonist Bay K 8644 (50 nM) was able to increase $J\text{Ca}_{\text{ms}}$ (Wadhwa and Care, 2000), whereas verapamil, a channel blocker, decreased net Ca absorption (Schröder et al., 1997; Wadhwa and Care, 2000), which could indicate the presence of L-type Ca^{2+} channels in the rumen epithelium. Although many epithelia use transient receptor potential vanilloid-type Ca^{2+} channels for Ca absorption (Hoenderop et al., 2005), L-type Ca^{2+} channels have been suggested to contribute additionally to intestinal Ca absorption (Morgan et al., 2003). Both the epithelial transient receptor potential vanilloid-type Ca^{2+} channels and L-type Ca^{2+} channels increase their conductance with alkaline pH (Pietrobon et al., 1989; Vennekens et al., 2001). An increase in mucosal Cl concentration in the rumen could thus stimulate the electrogenic part of ruminal Ca absorption via an increase in surface pH followed by a pH-dependent increase in luminal Ca^{2+} conductance (Figure 5).

For the electroneutral part of ruminal Ca absorption, a $\text{Ca}^{2+}/2\text{H}^+$ exchange mechanism has been suggested (Schröder et al., 1997; Wadhwa and Care, 2000). An increase in mucosal Cl concentration increases the activity of luminal $\text{Cl}^-/\text{HCO}_3^-$ exchange. This has been shown by an increase in bicarbonate secretion (Gäbel et al., 1991), an increase in surface pH (Leonhard-Marek et al., 2006), and an increased recovery of

isolated cells from an alkaline load in the presence of extracellular Cl (Bilk et al., 2005). Therefore, the Cl-dependent stimulation of electroneutral Ca absorption in the present study may be the consequence of an interaction between $\text{Cl}^-/\text{HCO}_3^-$ and $\text{Ca}^{2+}/2\text{H}^+$ exchange via intracellular pH or surface pH (Figure 5).

Effects of SCFA on Ca Absorption

A stimulatory effect of SCFA on Ca absorption across the sheep rumen epithelium has been reported by different groups (Schröder et al., 1997, 1999; Wadhwa and Care, 2000; Uppal et al., 2003) and has recently also been shown with rumen epithelia from slaughtered cattle (Ricken 2005). However, all these studies used buffer solutions in which 25 to 100 mM SCFA were replaced by gluconate in the control solution. The current experiment demonstrated that the addition of 58 mM gluconate to a standard rumen buffer decreased the concentration of free Ca^{2+} ions from 0.8 to 0.3 mM (Figure 3). This implies that the change from a control (gluconate) solution to an experimental (gluconate-free) solution may more than double the concentration of free Ca^{2+} ions. We therefore hypothesized that the stimulatory effect of SCFA might alternatively have been a blocking effect of gluconate on Ca flux rates under "control" conditions. When $J\text{Ca}_{\text{ms}}$ was measured in SCFA solutions without gluconate ($19.2 \pm 3.0 \text{ nmol cm}^{-2} \text{ h}^{-1}$, $n = 7$; buffer G; SCFA in Figure 4) or with gluconate ($13.4 \pm 0.7 \text{ nmol cm}^{-2} \text{ h}^{-1}$, $n = 12$; buffer B; low Cl in Figure 1), we found that gluconate could reduce Ca flux rates in the presence of SCFA ($P < 0.05$). In the same buffers, we also observed a huge difference in $[\text{Ca}^{2+}]_{\text{ion}}$, ranging from $0.77 \pm 0.01 \text{ mM}$ in the presence of SCFA alone ($n = 4$, buffer G) to $0.2 \pm 0.0 \text{ mM}$ in the presence of SCFA and gluconate ($n = 3$, buffer B). However, when we exchanged mannitol for a combination of Tris-OH and SCFA without increasing the free Ca^{2+} concentration (Table 1), we could confirm that SCFA were indeed able to stimulate net Ca absorption across the rumen epithelium because of an increase in $J\text{Ca}_{\text{ms}}$ (Figure 4). This means that the effects attributed to SCFA in the stimulation of Ca flux rates have been overestimated in previous studies, because gluconate should have decreased the control Ca flux rates in those experiments (Schröder et al., 1997, 1999; Wadhwa and Care, 2000; Uppal et al., 2003; Ricken 2005). However, the general conclusion of those studies was correct, that SCFA stimulate ruminal Ca absorption.

The current understanding of apical SCFA uptake in rumen epithelial cells involves the permeation of protonated SCFA, which are lipid soluble, and the uptake of SCFA^- anions in exchange for HCO_3^- (Gäbel et al., 2002). A stimulation of these pathways by an in-

crease in luminal SCFA concentration will provide protons to the cell interior and bicarbonate to the epithelial surface. This should stimulate a pH-dependent Ca^{2+} conductance as well as an apical localized $\text{Ca}^{2+}/2\text{H}^{+}$ exchanger, as discussed above (Figure 5). The SCFA could thus use the same mechanisms to stimulate Ca absorption, as discussed above for the stimulation of Ca absorption by luminal Cl^{-} ions. This conclusion is supported by a comparison of $J\text{Ca}_{\text{ms}}$ at a high-SCFA and low-Cl luminal concentration ($19.2 \pm 3.0 \text{ nmol cm}^{-2} \text{ h}^{-1}$, $n = 7$; Figure 4) with the corresponding flux rate at a high SCFA and high Cl^{-} luminal concentration ($20.3 \pm 1.4 \text{ nmol cm}^{-2} \text{ h}^{-1}$, $n = 6$; Figure 1). Considering these data, Cl seems to have no further effect on Ca absorption in the presence of SCFA.

Given the electrochemical gradients across the basolateral membrane (negative cell interior and low intracellular Ca^{2+} concentration), Ca extrusion to the blood side is an energy-dependent process. This Ca extrusion could be driven by Ca^{2+} -ATPase (Schröder et al., 1997) or by a $\text{Ca}^{2+}/\text{Na}^{+}$ exchange energized by the activity of the $\text{Na}^{+}/\text{K}^{+}$ -ATPase (Höller et al., 1988; Schröder et al., 1999). Intraepithelial metabolism of SCFA, especially of *n*-butyrate, is the predominant energy source in the rumen epithelium (Baldwin and Jesse, 1992). The SCFA therefore might additionally stimulate ruminal Ca absorption by the provision of ATP. Comparison of the Ca fluxes ($J\text{Ca}_{\text{ms}}$) in the presence of Cl ($14.5 \pm 1.4 \text{ nmol cm}^{-2} \text{ h}^{-1}$, $n = 8$; Figure 4) with those in the presence of Cl and SCFA ($20.3 \pm 1.4 \text{ nmol cm}^{-2} \text{ h}^{-1}$, $n = 6$; Figure 1) indicated an additional SCFA effect on $J\text{Ca}_{\text{ms}}$ in the presence of Cl ($P < 0.01$ between both groups), which would agree with the hypothesized metabolic stimulation (Figure 5).

Concentration of Free Ca^{2+} Ions in Physiological Buffer Solutions

In the freshly prepared solutions in the first experiment, $[\text{Ca}^{2+}]_{\text{ion}}$ amounted to 0.75 mM in the high-Cl solution (1.2 mM gluconate) and to 0.22 mM in the low-Cl solution (58.2 mM gluconate). Because the addition of gluconate reduced $[\text{Ca}^{2+}]_{\text{ion}}$, whereas the addition of Cl had no effect (Figure 3), the values cited above can be interpreted as a reduction in $[\text{Ca}^{2+}]_{\text{ion}}$ by 0.53 mM because of the 57 mM gluconate.

The association of Ca^{2+} ions with gluconate⁻ can be calculated as $K_{\text{ass}} = [\text{Ca-gluconate}^{+}]/(\text{Ca}^{2+} \text{ activity} \cdot [\text{gluconate}^{-}])$. For the measurements given above, $K_{\text{ass}} = 42.66$ and $\log K_{\text{ass}} = 1.63$. This value is in agreement with the $\log K_{\text{ass}}$ of 1.81 calculated by Christoffersen and Skibsted (1975). To estimate K_{ass} for sulfate and SCFA, we used the Ca^{2+} concentrations given in Table 2. The addition of 30 mM sulfate reduced $[\text{Ca}^{2+}]_{\text{ion}}$ from

Figure 6. Proportion of free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{ion}} \%$) that can be expected in a buffer solution with substantial concentrations of gluconate, sulfate, or short-chain fatty acids (SCFA). Proportions were calculated using the equations $K_{\text{ass}} = [\text{Ca-anion}^{+}]/([\text{Ca}^{2+}]_{\text{ion}} \cdot [\text{anion}^{-}])$ (from Christoffersen and Skibsted, 1975), and $[\text{Ca}^{2+}]_{\text{ion}} \% = [\text{Ca}^{2+}]_{\text{ion}} \cdot 100/([\text{Ca}^{2+}]_{\text{ion}} + [\text{Ca-anion}^{+}])$. The K_{ass} used for gluconate, sulfate, and SCFA were 42.66, 71.23, and 7.40, as shown in the Discussion section.

1.11 to 0.36 mM, resulting in a K_{ass} of 71.23; the addition of 60 mM SCFA reduced $[\text{Ca}^{2+}]_{\text{ion}}$ from 1.11 to 0.77 mM, resulting in a K_{ass} of 7.4. The same equation can be used to calculate the concentration of ionized Ca^{2+} that can be expected in solutions with increasing concentrations of gluconate, sulfate, or SCFA (Figure 6).

These calculations show that 20 mM gluconate will reduce the concentration of free Ca^{2+} ions by nearly 50%, whereas 100 mM gluconate will reduce $[\text{Ca}^{2+}]_{\text{ion}}$ further to only 20% of the original value in the absence of gluconate. A comparable reduction to 20% of the initial Ca^{2+} activity was previously reported by Kenyon and Gibbons (1977), who replaced 137 mM Cl with gluconate in Tyrode's solution. A 50% reduction in $[\text{Ca}^{2+}]_{\text{ion}}$ can also be expected with 15 mM sulfate or in the presence of about 100 mM SCFA (Figure 6).

These data shown in Figure 6 can be used as an estimate of $[\text{Ca}^{2+}]_{\text{ion}}$ reduction in comparable solutions. However, differences in ionic composition between solutions will affect $[\text{Ca}^{2+}]_{\text{ion}}$ (Günzel et al., 2005). In experiments in which $[\text{Ca}^{2+}]_{\text{ion}}$ is crucial, it should be measured instead of calculated.

Misestimation of Flux Rates and Possible Effects on Paracellular Permeability

Studies of nutrient and electrolyte transport mechanisms across the forestomach or hindgut epithelia often

use an SCFA gradient to mimic what happens in vivo. On the blood side, this amount of SCFA is frequently replaced by gluconate in the respective solutions, because gluconate gradients are considered to have only negligible effects on transport rates. However, in light of the reduced free Ca^{2+} concentration in a gluconate buffer, as shown above, such a strategy would lead to a misestimation of flux rates from the serosal to the mucosal side, if the flux rate is at all dependent on the available concentration of free Ca^{2+} ions. For Ca flux rates, this dependence is a direct one.

When the experiments in the present study were compared, there was a significant difference in $J\text{Ca}_{\text{sm}}$ between the 2 control conditions (gluconate and mannitol), which was correlated with a difference in free Ca^{2+} ions. All epithelia bathed in a gluconate solution ($[\text{Ca}^{2+}]_{\text{ion}} = 0.31 \text{ mM}$) on the serosal side showed a mean $J\text{Ca}_{\text{sm}}$ of $2.75 \pm 0.31 \text{ nmol cm}^{-2} \text{ h}^{-1}$ ($n = 18$), whereas epithelia bathed in the mannitol control solution ($[\text{Ca}^{2+}]_{\text{ion}} = 1.11 \text{ mM}$) showed a higher $J\text{Ca}_{\text{sm}}$ of $5.40 \pm 0.42 \text{ nmol cm}^{-2} \text{ h}^{-1}$ ($n = 20$, $P < 0.001$ vs. gluconate solution). This higher transport rate was not due to a higher transepithelial conductance in the second group of tissues. Tissues bathed in the mannitol solution even showed a lower G_t ($1.13 \pm 0.05 \text{ mS cm}^{-2}$) than those bathed in the gluconate solution ($G_t = 2.21 \pm 0.13 \text{ mS cm}^{-2}$, $P < 0.001$), which might point to a higher paracellular permeability in a gluconate solution because of the reduction in $[\text{Ca}^{2+}]_{\text{ion}}$.

Besides the increased $[\text{Ca}^{2+}]_{\text{ion}}$, an additional explanation for the higher $J\text{Ca}_{\text{sm}}$ in the mannitol control solution might be the lower Na concentration in these solutions. (To study the isolated effect of anion addition and end up with the same anion concentrations as in the first experimental series, we had accepted a difference in Na concentration between the 2 series as the smaller deviation.) Up to now, no structural or functional studies have been conducted on the mechanisms mediating basolateral Ca efflux from the rumen epithelium, but the observation that ouabain, a blocker of Na^+/K^+ -ATPase, reduced $J\text{Ca}_{\text{ms}}$ and increased $J\text{Ca}_{\text{sm}}$ (Höller et al., 1988) would support the involvement of $\text{Na}^+/\text{Ca}^{2+}$ -exchangers at the basolateral membrane. The existence of a significant electroneutral component of $J\text{Ca}_{\text{sm}}$ (Höller et al., 1988; present study) could further point to a transcellular Ca secretion via Ca exchangers operating in reverse mode. Thus, a reduction in serosal Na concentration together with the increase in $[\text{Ca}^{2+}]_{\text{ion}}$ might entail basolateral Ca uptake and an increase in $J\text{Ca}_{\text{sm}}$. However, we did not observe a decrease in $J\text{Ca}_{\text{ms}}$ under the conditions of a reduced Na concentration (cf. SCFA in Figure 4 vs. high Cl and SCFA in Figure 2), which argues against a major contribution of $\text{Na}^+/\text{Ca}^{2+}$ -exchange for transcellular Ca absorption or against its

operation in reverse mode despite the reduced Na concentration.

Although we did not find differences between $J\text{Ca}_{\text{sm}}$ measured with different solutions on the mucosal side, an altered $[\text{Ca}^{2+}]_{\text{ion}}$ on the mucosal side might likewise alter the chemical gradient and thereby have an effect on $J\text{Ca}_{\text{sm}}$, if the contribution of other variances is small. This might be one cause for the decreased electroneutral component of $J\text{Ca}_{\text{sm}}$ measured in a high-Cl buffer mucosal ($[\text{Ca}^{2+}]_{\text{ion, mucosal}} = 0.8 \text{ mM}$) compared with the value measured in a low-Cl (high-gluconate) solution on the mucosal side ($[\text{Ca}^{2+}]_{\text{ion, mucosal}} = 0.2 \text{ mM}$). The possible misestimation of J_{sm} flux rates attributable to gluconate solutions discussed here will, as a consequence, lead to a misestimation of net absorption rates, because $J_{\text{net}} = J_{\text{ms}} - J_{\text{sm}}$.

CONCLUSIONS

Chloride and SCFA stimulate active Ca absorption across the rumen epithelium. The available data suggest that this stimulation is due to pH effects on Ca^{2+} channels and on a putative $\text{Ca}^{2+}/2\text{H}^+$ exchange, with additional metabolic effects of SCFA. Sulfate had no effect on Ca absorption, whereas gluconate decreased Ca flux rates. The effect of gluconate could be correlated to a reduction in the concentration of free Ca^{2+} ions, which were also reduced by sulfate and SCFA anions. These data mean that ion-exchange experiments must be evaluated very carefully, because changes in $[\text{Ca}^{2+}]_{\text{ion}}$ in the presence of gluconate, sulfate, or SCFA might not only entail an underestimation of Ca flux rates, but might also have effects on other Ca^{2+} -dependent pathways.

Based on the effects of Cl and SCFA on ovine ruminal Ca absorption in the present study, and the effects of SCFA on bovine ruminal Ca absorption (Ricken, 2005), Ca salts included in the diets of dairy cows postpartum should be supplied as chlorides (in concentrations or formulations that are not caustic) or as salts of SCFA, preferably as propionate because of its additional glucogenic and energetic benefits.

ACKNOWLEDGMENTS

The technical assistance of M. Burmester and U. Dringenberg is greatly appreciated.

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