Rumen epithelial cells adapt magnesium transport to high and low extracellular magnesium conditions

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Abstract. A protein of ~ 70-kDa was identified as a candidate Na⁺/Mg²⁺ exchanger in rumen epithelial cells (REC). Melastatin-related Transient Receptor Potential 7 (TRPM7) and Magnesium Transporter 1 (MagT1) transcripts and, from them, encoded proteins were also detected. The regulation of these Mg transport pathways by extracellular [Mg] changes was the main focus of this study. Therefore, a 24-h pre-incubation of ovine REC in control (1.2 mM), low (0.12 mM)-Mg, and high (5 mM)-Mg medium was performed. Na⁺/Mg²⁺ exchangers, TRPM7 and MagT1 abundance and activity were investigated by Western blot analysis, flow cytometry, immunocytochemistry and fluorescence spectroscopic measurements of [Mg²⁺]i changes. Inhibitors were employed to differentiate Na⁺/Mg²⁺ exchanger-mediated (imipramine) and channel-mediated (cobalt(III)hexaamine, nitrendipine) Mg transport. Basal [Mg²⁺]i (0.40 ± 0.02 mM) was not influenced by pre-incubation in low- or high-Mg medium. However, compared with control REC (4.1 ± 0.7 μM/min), such cells showed reduced (2.8 ± 0.6 μM/min) or elevated (6.4 ± 0.9 μM/min) Mg extrusion rates that correlated with a decreased (25%) and increased (38%) expression of the putative Na⁺/Mg²⁺ exchanger protein, respectively. Low- and high-Mg pre-incubated REC were both characterized by an increased (30-40%) influx capacity. In low-Mg REC, the latter resulted mainly from a strong activation of the TRPM7-related transport component. The data thus clearly demonstrate the intrinsic regulation of REC transmembrane Mg transport.

Key words: sheep, rumen, Na⁺/Mg²⁺ exchanger, TRPM7, MagT1, mag-fura 2

Regulation of the free intracellular magnesium concentration ([Mg²⁺]i) and of whole body Mg homeostasis occurs through a variety of mechanisms involving pathways for Mg uptake, Mg efflux, and Mg exchange between intracellular compartments [2-5]. However, only a few Mg transport proteins, namely, the TRPM6 and TRPM7 channels of the Melastatin-related Transient Receptor Potential family and the mitochondrial channel MRS2 (Mitochondrial RNA Splicing member 2), have been identified at the molecular level so far [2, 6]. In addition, using a differential gene expression approach, the group of Quanume [7-11] described SLC41A1 and SLC41A2 (Solute Carrier family 41; subfamily A; members 1 and 2), MagT1 (Magnesium Transporter 1), ACDP 2 (Ancient Conserved Domain Protein; subtype 2), and the protein NIPA1 (Nonimprinted in Prader-Willi/Angelman) as putative Mg transporters. Based on the transport characteristics reported to date [9-13], it seems that only SLC41A1 mediates Mg efflux and that none of these proteins is identical to the Na⁺/Mg²⁺ exchanger. At a functional level, the latter has been demonstrated to mediate Mg
efflux in most cell systems investigated [14-18]. In our previous work with isolated ovine ruminal epithelial cells (REC) [5], we showed that a Na+/Mg2+ exchanger is the main Mg efflux mechanism in these cells, being responsible for about 98% of total Mg release. In ruminating animals, the required Mg is absorbed from the forestomachs by active transcellular mechanisms [19]. As shown by using the Ussing-chamber technique [20], a Na-dependent imipramine-sensitive mechanism is essentially involved in these processes. Moreover, the REC Na+/Mg2+ exchanger is an important mechanism for maintaining cellular Mg balance [5] by compensating for the marked Mg influx (37.5 to 42 μM/min) that is a characteristic of this Mg absorbing cell [21, 22]. In REC, as in other cell systems, [Mg2+]i can be assumed to be a cofactor for enzymes and signal-transduction proteins and to regulate bioenergetics, ion transport, growth, and proliferation [23-26]. In order to maintain [Mg2+]i in an optimal physiological range, Mg transport systems have to be under tight control. However, investigation of the modalities of the REC Mg transporter operation, regulation and interactions among each other is hindered by their unknown molecular identities.

Thus, the present study was performed to identify the main Mg influx pathways in REC at a molecular level. In addition, we investigated the effect of low and high extracellular Mg conditions on the expression and functional activity of TRPM7, MagT1 and the Na+/Mg2+ exchanger. This was done by using an anti-Na+/Mg2+ exchanger antibody prepared in our laboratory [27] and a combination of cell physiological approaches (fluorescence-spectroscopic measurement of the [Mg2+]i, flow cytometry, immunocytochemistry) and molecular techniques (RT-PCR, Western blot). The preliminary results were presented at the European Meeting on Magnesium and published in short form [1].

Materials and methods

Materials

Medium 199, trypsin, glutamine, antibiotics (gentamycin, nystatin, kanamycin, penicillin-streptomycin), and Dulbecco’s phosphate-buffered saline (DPBS) were purchased from PAN Biotech (Aidenbach, Germany). Fetal calf serum (FCS) and HEPes were obtained from Biochrom (Berlin, Germany) and Thermo Scientific (USA), respectively. Mag-fura 2-AM and pluronic acid were from Molecular Probes Inc. (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, USA).

Cell Culture

Primary cultures of REC were prepared as described by Galfi et al. [28]. Briefly, REC were isolated by fractional trypsinization and grown in Medium 199 containing 10% FCS, 1.36 mM glutamine, 20 mM HEPES, and antibiotics (50 mg/L gentamycin, 100 mg/L kanamycin) in an atmosphere of humidified air-5% CO2 at 38°C.

Experimental design

Experiments were performed between 5 and 6 days after seeding. On the day before a particular experiment, culture dishes were washed twice with warm DPBS and divided into three groups. Thereafter, to change the Mg status of cells, they were provided with fresh media supplemented with 1.2 mM (control), 5 mM (high-Mg), or 0.12 mM (low-Mg) Mg and then incubated at 38°C for 24 h. This pre-incubation medium was a custom-made (Biochrom, Berlin, Germany) Medium 199 with Earl’s salts containing no Ca/Mg or phenol red. Before use, 1.36 mM glutamine, 20 mM HEPES, 1.2 mM Ca and Mg as indicated above were added.

On the experimental day, some REC from each Mg group were loaded with mag-fura 2 to determine their Mg transport activity (see "Measurement of cytoplasmic Mg"), fixed with methanol for flow cytometric analysis of Na+/Mg2+ exchanger abundance (see "Flow cytometry"), or used to extract total protein (see "Western blot analysis").

Experiments to determine Mg efflux

The Mg efflux capacity was determined as the [Mg2+]i decrease over a 20-min period. Directly before the efflux experiment, REC were Mg-loaded by a 15-min incubation in divalent-free Hank’s balanced solution (HBS) supplemented with 1.2 mM Ca, 20 mM HEPES, 1.36 mM L-glutamine (HBSsup), and 5 mM Mg. Then, the remaining extracellular Mg was removed by washing the cells twice in a custom-made (Biochrom, Berlin, Germany) Na-free PBS (PBS-Na; No. 1825, but without Ca/Mg and with the Na being substituted with N-methyl-D-glucamine; NMDG). Thereafter, measurements were started by re-suspending REC in a custom-made (Biochrom, Berlin, Germany), completely Mg-free Medium 199 with Hank’s salts supplemented with 1.36 mM glutamine, 20 mM HEPES, 1.2 mM Ca.
Solution for Mg influx experiments

\([\text{Mg}^{2+}]_i\) increase was measured in the PBS-Na supplemented with 20 mM HEPES, 1.36 mM L-glutamine, and 5 mM Mg.

Measurement of cytoplasmic Mg

REC were rinsed twice with ice-cold divalent-free PBS, gently detached by incubation with HyQTase (15 min, at 38°C), centrifuged, washed twice in pre-incubation medium with the respective \([\text{Mg}]\) and once in HBS, and finally re-suspended in the last-mentioned solution. For the determination of \([\text{Mg}^{2+}]_i\), cells were loaded (25 min at 37°C) with 5 μM mag-fura 2-AM in the presence of pluronic acid. After centrifugation, cells were suspended in HBSsup, incubated for a further 30 min to allow for complete de-esterification, and washed twice in HBS before measurement of fluorescence. In influx experiments, these steps were performed with HBS supplemented with HEPES and L-glutamine only.

Intracellular ion concentrations were determined by measuring the fluorescence of the probe-loaded REC in a spectrofluorometer (LS-50 B, PerkinElmer, Wiesbaden, Germany) by using the fast-filter accessory, which allowed fluorescence to be measured at 20-ms intervals with excitation for mag-fura 2 at 340 and 380 nm and emission at 515 nm. All measurements were made at 37°C in a 3-mL cuvette containing 2 mL cell suspension (10% cytocrit) under stirring.

\([\text{Mg}^{2+}]_i\) was calculated from the 340/380-nm ratio according to the formula of Grynkiewicz et al. [29] by using a dissociation constant of 1.5 mM for the mag-fura-2/Mg complex. The minimum (Rmin) and maximum (Rmax) ratios were determined at the end of each experiment by using digitonin. Rmax was measured after the addition of 25 mM MgCl2 in the absence of Ca, and Rmin was obtained by addition of 50 mM EDTA, pH 7.2, to remove all Mg from the solution.

Determination of intracellular cAMP concentration

The intracellular cAMP concentration ([cAMP])i was determined in REC (10⁶ cells/mL) seeded in 96-well plates (100 μL per well) and incubated overnight in an FCS-free Medium 199. On the next day, the 24-h pre-incubation of REC with media containing 1.2, 5, or 0.12 mM Mg was initiated as described above. Afterwards, [cAMP]i was measured by use of an enzyme-linked immunoassay system (Amersham Pharmacia Biotech, UK) according to the protocol of the manufacturer.

RNA isolation and reverse-transcription polymerase chain reaction (PCR)

Total ovine REC RNA was isolated by use of the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol, and its integrity was examined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). Total RNA (100 ng) and oligo (dT)₁₂ primers were used to synthesize cDNA from poly A-containing mRNA with the iScript cDNA synthesis kit (BioRad, Hercules, USA). The cDNA (TRPM6/7 and MagT1) was amplified by PCR (peqSTAR, PEQLAB Biotechnologie, Erlangen, Germany) with HotStar HiFidelity Polymerase (Quiagen, Hilden, Germany).

The following gene-specific primers were used for PCR amplification (the identity of the particular gene is denoted in the name of each primer):

- (1) bTRPM7-forw-(588-608); IVF-FU#501b: 5′-TTGGCCAGAGTGAAGCAGTT-3′;
- (2) bTRPM7-rev-(745-764); IVF-FU#478b: 5′-TTTCCAACAGTGCCATCATC-3′;
- (3) hMagT1-forw-(540-559); IVF-FU#689b: 5′-GCGACAGAACTGTGCTCA-3′;
- (4) hMagT1-rev-(749-768); IVF-FU#690b: 5′-TATGGGCATATGGTGGTCCT-3′;
- (5) hTRPM6-forw(P1)-(4356-4375); IVF-FU#774b: 5′-TGGCAACTGAACAGGACATC-3′;
- (6) hTRPM6-rev(P1)-(4811-4830); IVF-FU#775b: 5′-CGGAGAGGATTGATCCAAAA-3′;
- (7) hTRPM6-forw(P2)-(4437-4456); IVF-FU#776b: 5′-GGATGAACCCAAGGAAAAG-3′;
- (8) hTRPM6-rev(P2)-(4831-4850); IVF-FU#777b: 5′-TAGCGAAGGGCCTGTATCTG-3′.

For all three genes, optimal amplification was obtained in a reaction volume of 50 μL and with the following PCR program (35 cycles): denaturation at 94°C for 15 s, annealing at 53°C (TRPM6) or 55°C (TRPM7 and MagT1), and elongation at 72°C for 1 min.

Ethidium-bromide-stained PCR-products were electrophoretically resolved on a 2% agarose gel and visualized in ChemilmagerTM 5500 (Alpha Innotech, San Leandro, USA). The PCR-amplified TRPM7, MagT1, and TRPM6 fragments were sequenced (AGOWA, Berlin, Germany) for identity-verification purposes.
Antibodies

The monoclonal mouse anti-Na\(^+\)/Mg\(^{2+}\) exchanger antibody (mab) used in this study was raised against the porcine erythrocyte Na\(^+\)/Mg\(^{2+}\) exchanger in our laboratory and has been shown to react specifically with the protein in REC [5, 27]. The hybridoma cultures were established in RPMI1640 medium (PAN Biotech, Aidenbach, Germany) containing 10% FCS. During days 2-3, hybridomas were stepwise switched to serum-free medium for mab production (HyQ ADCF-Mab, HyClone, Bonn, Germany). Culture supernatants containing the produced mabs were sampled for 4-6 days. Thereafter, supernatants were purified and concentrated by ultracentrifugation in Vivaspin Centrifugal Concentrators (Vivaspin 15R, Vivascience, Hannover, Germany). Centrifugation was performed at 3 000 \(\times\) g for 30 min. After dilution of the concentrated sample in PBS (1:1), a second ultrafiltration step was performed. The IgG concentration was then determined by using a Nanodrop photometer (Implen GmbH, München, Germany) and set to about 1 mg/mL with PBS containing 0.5% bovine serum albumin (BSA) and 0.02% Na-azide. Aliquots were subsequently stored at -20°C until use. A rabbit anti-TRPM7 antibody including a GH3 cell line protein lysate as control antigen (ACC-047, lot AN-01) was obtained from Alamone Labs (Jerusalem, Israel). The rabbit anti-MagT1-antibody was a gift from Dr. G. Quamme (Department of Medicine, University of British Columbia, Vancouver, Canada) and the mouse anti-β-actin antibody was purchased from Sigma (St. Louis, MO, USA). For Western blotting, secondary anti-mouse or anti-rabbit IgG antibodies coupled with horseradish peroxidase (HRP; Sigma, St. Louis, MO) were used. Alexa fluor 488-conjugated anti-mouse or anti-rabbit IgG antibodies obtained from Invitrogen (Paisley, UK) or Molecular Probes (Eugene, OR) were used for immunocytochemistry and flow cytometric experiments.

Western blot analysis

For Western blot analysis, total proteins from washed REC were extracted by use of the M-PER Mammalian Protein Extraction Reagent (Pierce, Bonn, Germany), complemented with a protease inhibitor cocktail (Pierce, Bonn, Germany). The protein concentration was determined by means of the Bradford assay (Sigma, St. Louis, MO, USA). Protein samples (40 to 80 μg) were separated by SDS (10%)-polyacrylamide gel electrophoresis and subsequently blotted to polyvinylidene fluoride (PVDF) membranes. After transfer, membranes were blocked with a 3% solution of non-fat dry milk in TRIS-buffered saline (pH 7.5) containing 0.05% Tween 20 (TBS-T) for 1 h. Following blocking, membranes were incubated at 4°C with the primary anti-Na\(^+\)/Mg\(^{2+}\) exchanger antibody (1:200 dilution; 0.25 μg/mL) and with the mouse anti-β-actin antibody (0.5 μg/mL, a gel-loading control) overnight, washed three times for 10 min in TBS-T, incubated for 1 h with HRP-conjugated secondary antibody (diluted 1:10,000), and finally washed three times for 10 min in TBS-T. Bands were visualized in Chemilumager TM 5500 (Alpha Innotech, San Leandro, CA, USA) with the Rotilumin reagents (Roth, Karlsruhe, Germany) or ECL Plus Western Blotting detection system (GE Healthcare/Amersham). Relative intensities of the band-densities were determined by analysis with NIH ImageJ 1.410 software (US National Institutes of Health, Bethesda, MD, USA).

Flow cytometry

Methanol-fixed REC were incubated overnight at 4°C with anti-Na\(^+\)/Mg\(^{2+}\) mab (5 μg/mL) in 10 mM PBS with 0.2% BSA and 1 mM EDTA, pH 7.3). After being warmed to room temperature, cells were washed twice in PBS-EDTA and incubated for 1 h in a 200-fold dilution (0.5 μg/mL) of Alexa fluor 488-conjugated anti-mouse-IgG(F(ab’s))\(_2\) (Molecular Probes, Eugene, OR). The anti-Na\(^+\)/Mg\(^{2+}\) exchanger antibody was omitted from control incubations. After a further two washes in PBS-EDTA, quantitative analysis of cellular fluorescence was carried out by flow cytometry to analyze the cells simultaneously according to size, granularity, and Na\(^+\)/Mg\(^{2+}\) exchanger abundance (portion of protein-expressing cells and relative fluorescence intensity per cell). TRPM7 and MagT1 abundance was determined in the same way, but the specific (5 and 4 μg/mL) and secondary (Alexa fluor 488-conjugated anti-rabbit-IgG, 0.5 μg/mL) antibodies were used. Flow cytometric analysis was performed as described previously [5]. Briefly, an argon-laser-equipped flow cytometer (Beckmann Coulter-XL, Krefeld, Germany) was used to record emissions of multiple fluorescence (green, orange, red) excited at 488 nm (counting 5 000 cells). Particle size was calibrated by using standard beads (Coulter). Cells of interest were identified by:

- establishing a histogram on the basis of cell size and granularity;
- establishing the fluorescence histogram;
- projecting the fluorescence into the size-granularity histogram. Afterwards, the cells were gated, and the portion of fluorescent cells and their fluorescence intensity were automatically computed.

Immunocytochemistry

REC (2 × 10^6 cells/mL) were grown on sterile glass cover slips (Neolab, Germany) for 24 to 48 h. Thereafter, the 24-h pre-incubation was performed as described above. After being rinsed twice with PBS, REC were fixed in methanol (10 min at -20°C). If not otherwise stated, all the following steps were carried out at room temperature. After two PBS washes, cells were permeabilized in 0.3% Triton X-100 in PBS with 7% goat serum (Dianova, Hamburg, Germany) for 10 min and again rinsed three times with PBS. Non-specific binding of IgG was suppressed by incubation of specimens with 7% goat serum in PBS for 20 min. Subsequently, cells were rinsed with PBS (three times for 5 min) and then incubated overnight at 4°C with the primary anti-Na+/Mg²⁺ exchanger antibody (0.25 mg/mL solved in PBS with 1% BSA; PBS-BSA). After being rinsed three times with PBS, cells were incubated for 2 h with the secondary, Alexa Fluor-488-labeled goat anti-mouse IgG1 (γ1) antibody (1:200 in PBS-BSA). After three PBS washes, nuclei were counterstained with 300 nM 4,6-diamidino-2-phenylindole (DAPI) in S-buffer (containing: 75 mM KCl, 3 mM MgSO₄·7H₂O, 1 mM EGTA, 0.2 mM dithiothreitole, 10 mM imidazol, 1 μg/mL aprotinin, 0.1 mM phenylmethane sulfonyl-fluoride). Cover slips were then mounted with 30 μL mounting medium (Dianova, Hamburg, Germany). Digital images were acquired by using a fluorescence microscope Olympus IX50 (Hamburg, Germany) and MetaMorph version 7.5.2.0 and AutoDeblur version 1.4.1 software (Visitron Systems GmbH, Puchheim, Germany).

Statistical analysis

If not otherwise stated, data are presented as means ± standard error (SE). Significance was determined by Student’s t-test or the paired t-test 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 (Jandel Scientific).

Results

[Mg²⁺]ᵢ of REC after a 24-h pre-incubation in media with various extracellular [Mg²⁺]

The basal [Mg²⁺]ᵢ of Mag-Fura 2-loaded REC was measured in Mg-free media after a 24-h incubation of cells in media with normal (1.2 mM), high (5 mM), or low (0.12 mM) [Mg]. As demonstrated in figure 1, [Mg²⁺]ᵢ was unaltered in low- and high-Mg REC (0.49 ± 0.04 and 0.41 ± 0.02 mM) versus control (0.40 ± 0.03 mM). This result indicates that mechanisms are present enabling cells to maintain an optimal [Mg²⁺]ᵢ under various conditions. Therefore, in the next experimental step, we determined the efflux capacity of differently pre-incubated REC.

[Mg²⁺]ᵢ change in differently pre-incubated REC

In contrast to other cell systems [30, 31], REC are known to have a high basal Mg influx capability [21]. For this reason, there was no need to load the cells with Mg by using the ionophore A 23187 as in our previous study [5]. To prepare REC for the following efflux experiment, it was sufficient to incubate them for 15 min in high-Mg loading solution ([Mg²⁺]ₑ = 5 mM). Figure 1 shows that in control and treatment groups a significant [Mg²⁺]ᵢ increase of about 158 ± 26 μM was induced by this treatment.

Directly after loading, efflux experiments were started by suspending REC in completely Mg-free but Na-containing media. The Mg efflux capacity was measured as a decrease of the [Mg²⁺]ᵢ over a 20-min period. Previously, the latter has been demonstrated to reflect Na-dependent Mg extrusion from REC [5]. Typical original traces of the [Mg²⁺]ᵢ decrease observed during the incubation of control, high-, and low-Mg pre-incubated REC in totally Mg-free Na media are given in figure 2A. Clearly, the [Mg²⁺]ᵢ decrease was faster and stronger after pre-incubation in high-Mg medium and reduced in low-Mg REC, when compared with control cells. Data from all experiments are summarized in figure 2B. For low-Mg conditions only results from measurements with detectable efflux (n = 10 of 12) were used for calculations. Compared with control cells (4.1 ± 0.7 μM/min), REC pre-incubated in low- and high-Mg medium showed reduced (2.8 ± 0.6 μM/min) and accelerated Mg extrusion rates (6.4 ± 0.9 μM/min), respectively.
Basal intracellular \([\text{cAMP}]\) and cAMP effect on the \([\text{Mg}^{2+}]\) change observed in differently pre-incubated REC

An increase of the intracellular cAMP concentration (\([\text{cAMP}]\)) has been shown to stimulate the Na-dependent Mg efflux in REC [5]. Therefore, a changed Mg efflux capacity of REC after pre-incubation in media with different \([\text{Mg}^{2+}]\) may result from the modulation of the basal \([\text{cAMP}]\) of such cells. However, REC mean \([\text{cAMP}]\), amounted to 5.1 ± 0.5 fmol/μg protein (n = 36), and no influence of the extracellular Mg status was detectable (figure 2C).

Next, we wished to know whether the pre-incubation in low- or high-Mg solutions had any effect on the response of REC to cAMP-stimulation. We therefore repeated the above-described efflux experiments in the presence of the cell membrane-permeable cAMP analog db-cAMP (100 μM). The data from these experiments are summarized in figure 2B and show that the observed \([\text{Mg}^{2+}]\) decrease was always stronger after db-cAMP application and was stimulated by 64 ± 18, 109 ± 39, and 304 ± 92% in control, high-, and low-Mg REC, respectively.

Influence of \([\text{Mg}^{2+}]\) on the abundance of REC putative Na\(^{+}/\text{Mg}^{2+}\) exchanger

To test whether a distinct Na\(^{+}/\text{Mg}^{2+}\) exchanger protein expression could cause the changed efflux capacity, we used Western analysis and flow cytometry (figure 3). As in previous experiments with REC and other cell systems [5], our anti-Na\(^{+}/\text{Mg}^{2+}\) antibody labeled a single protein with an apparent molecular weight of 70 kDa as the candidate Na\(^{+}/\text{Mg}^{2+}\) exchanger protein (figure 3A). Flow cytometric analysis confirmed the presence of this protein in primary cultured REC (figure 3C), with an average of 80 ± 3% cells being positive for Na\(^{+}/\text{Mg}^{2+}\) exchanger.
Typical results of Western blot analysis of whole cell protein extracts derived from differently pre-incubated REC are presented in figure 3B (inset). Downstream densitometric analysis, which is summarized in figure 3B, revealed that the amount of the detected protein was decreased (9.9 ± 1.3%) after pre-incubation in the low-Mg medium or increased (17.7 ± 2.6%) after pre-incubation in high-Mg medium compared with control (REC pre-incubated in medium containing 1.2 mM Mg).

Densitometric analysis conducted for immunolabeled β-actin (42 kDa, negative experimental control and loading control) did not show any [Mg^{2+}]_i-dependent variation in its abundance (figure 3B). Flow cytometric analysis (figure 3C) confirmed that, compared with control conditions, a decreased (-24.8 ± 3.7%) or increased (36.2 ± 6.4%) Na^+/Mg^{2+} exchanger protein amount per single cell was observed after a 24-h incubation of REC in low- and high-Mg medium, respectively.
Figure 3. Detection of the putative Na⁺/Mg²⁺-exchanger protein in ovine REC and analysis of the effect of a 24-h pre-incubation in low or high extracellular [Mg] on its expression. A) Immunoblot of the Na⁺/Mg²⁺-exchanger in sheep REC. Ovine REC protein lysates were electrophoresed on a 10% polyacrylamide gel and blotted onto PVDF membranes. Membranes were stained with the purified anti-Na⁺/Mg²⁺ exchanger antibody (0.25 μg/mL) and bound antibodies were visualized by using the ECL Plus Western Blotting detection system (GE Healthcare/Amersham). The anti-Na⁺/Mg²⁺-exchanger antibody detected a band at the expected size of approximately 70 kDa (lane 1). No band appeared after omission of the primary antibody (lane 2 = negative control). B) Relative abundances of Na⁺/Mg²⁺ (~70 kDa) exchanger in REC incubated in medium containing low (0.12 mM) or high (5 mM) [Mg]. Densitometric analysis was performed using ImageJ freeware. The percentage increase or decrease of Na⁺/Mg²⁺ exchanger abundance was calculated as difference between its abundance in low- or high-Mg incubated REC related to its abundance in cells incubated in medium containing normal [Mg] (1.2 mM). For comparison, the relative abundances of β-actin (42 kDa) in REC incubated under the same conditions are given as well. Data were averaged out of 9 independent experiments. Inset: Representative example of a Na⁺/Mg²⁺ exchanger Western blot, used for the ImageJ analysis. C) Flow cytometric analysis of the effect of low and high extracellular [Mg] on the cellular abundance of the Na⁺/Mg²⁺ antiporter protein. Left part: The number of fluorescence-positive cells (ordinate) was plotted against the fluorescence intensity per cell (channel number, abscissa). The left (obtained by incubation of a cell aliquot only with fluorogenic antibodies) and right (obtained by incubation of a cell aliquot with anti-Na⁺/Mg²⁺ exchanger and fluorogenic antibodies) peaks
represent controls and Na+/Mg2+ exchanger-positive cells, respectively. The right shift of the channel number after incubation with the primary anti-Na+/Mg2+ exchanger antibody shows its specific binding to this protein. Right part: the percent change of the fluorescence intensity per cell (representing a measure of the amount of Na+/Mg2+ exchanger protein per single cell) is given in relation to control conditions (100%). REC pre-incubated in low or high extracellular [Mg] show a significantly reduced or increased Na+/Mg2+ exchanger expression, respectively. Bars represent results from 10 single experiments; * p < 0.05 vs control.

Epithelial and cellular localization and abundance of the putative Na+/Mg2+ exchanger

For a better understanding of the following data, the morphology of the rumen epithelium is shown in figure 4A. It can be seen that, compared with the single layer of renal or intestinal epithelia, the rumen epithelium has a more complex multilayered structure. Starting from the blood side, four distinct cell layers, namely, the stratum (str.) basale, the str. spinosum, the str. granulosum, and the keratinized str. corneum, can be distinguished (figure 4A). Three different REC fractions obtained by trypsination as described in "materials and methods" and representing 1) mainly cells from the stratum basale, 2) mainly cells from str. spinosum and lower str. granulosum, and 3) mainly cells from stratum granulosum were analyzed for Na+/Mg2+ exchanger abundance using flow cytometry. The composition of the cell fractions was evaluated by microscopy but it has to be mentioned that every fraction contained some cornified cells not involved in epithelial transport processes. As shown in figure 4B, the Na+/Mg2+ exchanger was enriched in cells of the stratum basale (45 ± 9% of positive cells). A progressive reduction of the number of Na+/Mg2+ exchanger-positive REC was observed in the stratum spinosum (29 ± 5%) and stratum granulosum (24 ± 4%).

Next we investigated the distribution and abundance of the putative REC Na+/Mg2+ exchanger protein by immunocytochemistry, and examples of characteristic staining patterns are given in figure 5. The Na+/Mg2+ exchanger was localized in the cell membrane or in its close vicinity, although cytoplasmic staining was also observed (figure 5). The latter seemed to increase if REC had been incubated in the high-Mg medium. Corresponding to results from the functional, Western blot, and flow cytometric experiments, the expression level of the putative Na+/Mg2+ exchanger was considerably reduced in low-Mg pre-incubated REC (figure 5A), and more protein was labeled after pre-incubation in high-Mg medium (figure 5C).

Role of other Mg transport systems

To date, functional studies performed at the tissue and cell levels [21, 22, 32] suggest the existence of at least two different Mg uptake mechanisms in REC. Although their molecular identity is not known, candidates are the recently described TRPM6, TRPM7, and MagT1 Mg channels/transporters [4, 9, 33], which have been shown to be influenced by Mg status [9, 34]. PCR revealed the TRPM7 and MagT1 transcripts in REC (figure 6A). The results were confirmed by sequencing the products. The sequences attained were compared with the ovine (TRPM7) or human (MagT1) sequences that had been used for the primer design, yielding an identity of 100 and 99.6%, respectively. In addition, flow cytometric analysis showed the presence of TRPM7 in 93 ± 2% and of MagT1 in 80 ± 10% of these cells. In contrast, we were not able to identify TRPM6 transcripts with specific primer pairs (PP1 and PP2; figure 6A) in REC. As a control, PP1 and PP2 were used to detect TRPM6 transcripts in Caco-2 (human epithelial colorectal adenocarcinoma) cells. In this cell system the presence of the TRPM6 transcript tested positive (figure 6A).

We next evaluated whether a pre-incubation in Mg-deficient or high-Mg medium resulted in activity changes of Mg influx pathways. To test this possibility, influx experiments were performed in the absence of Na ([Na+]i > [Na+]e) and the presence of 5 mM Mg ([Mg2+]i < [Mg+]e) in the extracellular solution. Under such conditions, some of the Mg uptake is mediated via the Na+/Mg2+ exchanger working in the reverse mode [5, 32]. Known unspecific inhibitors of the Na+/Mg2+ exchanger (imipramine [32] and of channel-mediated Mg influx (Co(III)hexaamine [2, 13]) were used to differentiate between transport components. In addition, cell samples were analyzed for the abundance of TRPM7 and MagT1 by using Western analysis and flow cytometry.

The results of the functional experiments are given in figure 6B. Irrespective of the [Mg] of the pre-incubation medium, the initial [Mg2+]i of REC amounted to 0.68 ± 0.04 mM in these experiments.
In all three treatment groups, a significant $[\text{Mg}^{2+}]_i$ increase occurred during the 20-min incubation period. However, compared with control cells ($19.8 \pm 1.4 \mu\text{M/min}$), low- and high-Mg pre-incubated REC were both characterized by an increased influx capacity amounting to $25.8 \pm 1.7$ and $26.4 \pm 2.8 \mu\text{M/min}$, respectively (figure 6B). Application of imipramine ($250 \mu\text{M}$), Co(III)hex ($1 \text{mM}$), or of a combination of both inhibitors led to a reduced $[\text{Mg}^{2+}]_i$ increase in all groups of REC, thereby reflecting the existence of channel-mediated and of Na-dependent components of Mg transport. In agreement with the observed changes in $\text{Na}^+$/Mg$^{2+}$ exchanger expression, the imipramine effect was lowest ($-4.8 \pm 2.0 \mu\text{M/min}$) in REC pre-incubated in the 0.12 mM-Mg medium and highest ($-13.4 \pm 2.6 \mu\text{M/min}$) in cells pre-incubated in the high-Mg medium. However, the response of low-Mg REC to imipramine was not significantly different from that ($-5.7 \pm 2.3 \mu\text{M/min}$) of control cells. In the latter, the Co(III)Hex-induced decrease in the Mg uptake rate amounted to $2.5 \pm 0.5 \mu\text{M/min}$. Interestingly, the inhibitor effect was stronger in both experimental groups, and the Mg uptake rate was reduced by $12.2 \pm 2.8$ and $7.7 \pm 0.8 \mu\text{M/min}$ in low- and high-Mg pre-incubated REC, respectively. No additive effects of Co(III)Hex and imipramine were observed.

To evaluate the TRPM7 and MagT1 protein content in REC, immunoblotting was performed. As shown in figure 6C, Western analysis of total REC homogenate revealed major bands at the 160- to 170-kDa and 38-kDa positions, corresponding to TRPM7 and MagT1, respectively. A total protein lysate derived from GH3 cells (Alamone Labs, Jerusalem, Israel) was used as a positive control. Interestingly, pre-incubation in media with different $[\text{Mg}]_e$ had a slight influence on the protein expression of TRPM7 only (figure 6C). However, compared with control conditions, a decreased or increased MagT1 ($-35 \pm 10\%$; $20.1 \pm 9.5\%$) abundance was observed after the 24-h incubation of REC in low- and high-Mg medium, respectively. These latter results indicate the importance of obtaining information on the functional activity of MagT1 in control REC and in cells pre-incubated under low- or high-Mg conditions. Goytain and Quamme [9] have shown that the 1,4-dihydropyridine analog nitrendipine is an effective inhibitor of MagT1-related currents. Therefore, we studied the effect of this blocker ($50 \mu\text{M}$) on REC Mg influx measured over a 20-min period after reversing the transmembrane Na gradient ($[\text{Mg}]_e = 5 \text{ mM}$). The results are summarized in figure 7. Again, low- ($36 \pm 4 \mu\text{M/min}$) and high-Mg ($30 \pm 2 \mu\text{M/min}$) pre-
incubated REC showed an increased Mg uptake compared with the control cells (21 ± 0.6 μM/min). The inhibitor application reduced the Mg influx by 17 ± 3, 10 ± 1, and 16 ± 2 μM/min in low-Mg, control, and high-Mg REC, respectively.

Discussion

The plasma membrane Na⁺/Mg²⁺ exchanger is thought to be a key element in the regulation of both organism and cellular Mg homeostasis [35-
Figure 6. Expression of TRPM7 and MagT1 and Mg\(^{2+}\) influx capacity of differently pre-incubated REC. A) EtBr-imaging of the MagT1(230 bp)-; TRPM7(178 bp)- and TRPM6(476 bp-PP1; 415 bp-PP2)-DNA fragments, which were amplified from total REC or Caco-2 cells (as indicated) cDNA and electro-separated in an 1.5% agarose gel. The respective sizes of the fragments were compared to 100 bp DNA ladder (extended version; Carl Roth, Karlsruhe, Germany). B) Mg influx capacity of control, low-, and high-Mg REC and inhibitory effect of imipramine and/or cobalt(III)hexaamine. Changes in the intracellular Mg concentration ([Mg\(^{2+}\)]\(_i\)) of REC suspended in Mg-containing (5 mM), completely Na-free medium ([Na]\(_i\) < [Na]\(_e\) = influx conditions; Note that the Na\(^+\)/Mg\(^{2+}\) exchanger works in the reverse mode under these experimental conditions) were measured. In parallel, measurements were made in the same medium after addition of 1 mM Co(III)Hex and/or of 250 \(\mu\)M imipramine. Values are means ± SE from 8 single experiments; * p < 0.05 vs control. C) Immunoblot and flow cytometric analysis of the effect of low and high extracellular [Mg] on TRPM7 and MagT1 abundance in sheep REC. Left panel: Ovine REC protein lysates from low-Mg (lane A), control (lane B), and high-Mg (lane C) REC were used. For TRPM7, total protein lysates from the GH3 cell line were used as positive control (lane D). Right panel: Results of flow
This is supported by the results of this study showing that the transporter functional activity and its expression are quantitatively altered by extracellular Mg deficiency or overload.

\[ [\text{Mg}^{2+}]_i \text{ is regulated by a changed Mg efflux capacity} \]

The modulation of the extracellular \([\text{Mg}^{2+}] \) induced no significant change of the REC basal \([\text{Mg}^{2+}]_i \) measured in Ca/Mg-free medium. It ranged from 0.40 ± 0.03 to 0.49 ± 0.04 mM and was thus consistent with the values of 0.37 ± 0.05 and 0.54 ± 0.08 mM seen in our previous studies [5, 21]. This has led us to hypothesize that transmembrane Mg transport is adapted to perpetuating the physiological \([\text{Mg}^{2+}]_i \).

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The REC \(\text{Na}^+/\text{Mg}^{2+}\) exchanger has been shown to mediate 98% of Mg efflux [5], and its inhibition induces a marked increase in the cytosolic \([\text{Mg}^{2+}]_i \) [32]. Therefore, it represents a good candidate for playing an important role in \([\text{Mg}^{2+}]_i \) regulation. Our data show that the Mg efflux capacity of REC has indeed been modulated by \([\text{Mg}]_e \) changes. Cells pre-incubated in Mg-deficient and high-Mg medium are characterized by a decreased (32%) and accelerated (56%) Mg efflux rates, respectively, when compared with controls. Interestingly, Feillet-Coudray et al. [39] observed a decreased Mg efflux in erythrocytes obtained from mice fed a Mg-deficient diet. To examine the regulation of Na-dependent Mg efflux in the renal epithelial cell line NRK-52E, Ikari et al. [40] cultured them for 1-2 days in media con-
Our results clearly show that the Na\(^+\)/Mg\(^{2+}\) homeostasis is part of the system regulating REC Mg transport through transcription- and translation-dependent processes to maintain Mg balance. Epithelial cells from the renal tubule show an intrinsic adaptation to diminished extracellular magnesium involving an increased expression of pathways related to Mg uptake and/or their translocation to the cell membrane [41, 11, 7]. Thus, we investigated the influence of low and high medium [Mg\(^{2+}\)] on the expression of the 70-kDa protein representing the candidate Na\(^+\)/Mg\(^{2+}\) exchanger.

A change of the Na\(^+\)/Mg\(^{2+}\) exchanger expression is part of the system regulating REC Mg homeostasis

Our results clearly show that the Na\(^+\)/Mg\(^{2+}\) exchanger protein is quantitatively altered following changes in extracellular Mg. We used three different methods, namely immunobloting, flow cytometry, and immunocytochemistry, and obtained concordant results showing that the amount of Na\(^+\)/Mg\(^{2+}\) exchanger protein is elevated after exposure of REC to high-Mg conditions and is reduced by incubating them in the low-Mg solution. Flow cytometric analysis enables real quantification of the effects and revealed a 36% and 25% increase and decrease in the Na\(^+\)/Mg\(^{2+}\) exchanger abundance per single cell, respectively. As all methods give similar findings, we conclude that a changed Na\(^+\)/Mg\(^{2+}\) exchanger expression is the main mechanism in the homeostatic up- or down-regulation of Na-dependent Mg transport. Excess extracellular Mg can be assumed to induce an increased Mg efflux via the increased abundance of the Na\(^+\)/Mg\(^{2+}\) exchanger in the REC cell membrane and an elevated activity of the protein. First, this protects cells against Mg overload; and second, it generates a driving force to maintain the Mg influx into this absorbing cell [42, 21]. The way that this extracellular [Mg] sensing works is not yet clear. The Ca/Mg-sensing receptor might be involved because an increased functional activity of a Na-independent Mg efflux has been demonstrated after its stimulation [43].

In addition to intrinsic mechanisms [9, 11, 41], extracellular stimuli can affect Mg transport [34, 43-46]. Various hormones and other factors are known to activate the Na\(^+\)/Mg\(^{2+}\)-exchanger-related Mg efflux via protein kinase C [47, 48], PI 3-kinase [49], or the cAMP-protein kinase A [30, 50, 51] pathways. The last-mentioned specifically triggers Na-dependent Mg release from REC [5] and increases transcellular Mg absorption across the rumen epithelium [20]. Measurements of REC [cAMP], have shown that the concentration of this second messenger is not changed after modification of the transmembrane Mg gradient. In consequence, we tested whether Na\(^+\)/Mg\(^{2+}\) exchanger sensitivity to intracellular cAMP was influenced by pre-incubation of REC in media with reduced or elevated [Mg].

Sensitivity of the Na\(^+\)/Mg\(^{2+}\) exchanger to an intracellular [cAMP] increase is maintained

As in our previous studies [5, 32], we used db-cAMP, a cell permeant cAMP analog to modulate the [cAMP]\(_i\) of REC. The applied dosage of 100 μM has been shown to increase the [cAMP]\(_i\) about 12-fold [5]. Independently of the [Mg] of the pre-incubation medium, the stimulation of REC with db-cAMP results in a markedly increased Mg efflux. In agreement with investigations on erythrocytes [52], the stimulatory effect of cAMP is lower in control (64%) and high-Mg (109%) REC, than in low-Mg cells (304%). Following phosphorylation of the transport protein, cAMP is known to augment the affinity of the Na\(^+\)/Mg\(^{2+}\) exchanger for intracellular Mg and to stimulate Mg extrusion from non-loaded cells [30, 50, 52]. The latter also provides an explanation for the lower 55% stimulation seen after db-cAMP application of artificially (A23187) Mg-loaded REC [5]. A strong cAMP-related stimulation of Mg efflux in low-Mg pre-incubated REC might be of pathophysiological importance if this leads to a further loss of intracellular Mg, e.g., by the extrusion of Mg that has been mobilized from intracellular buffers and organelles.

The keratinizing epithelium lining the rumen has a very complex multilayer structure. In contrast to monolayer epithelia, functional polarization and thus generation of transport gradients is realized by dominant location of transport proteins to special cell layers. Like the ruminal Na/K-ATPase [53],

the Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger can be found in the cell membrane of most non-keratinized REC but is particularly expressed in the basal cells. As the basal cell layer corresponds to the basolateral membrane of monolayer epithelia, this result is in accordance with the postulated role of the ruminal Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger for transepithelial Mg absorption. However, a profound investigation of the Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger localization in the rumen epithelium is beyond the scope of the present study.

**TRPM7 and MagT1 are the main Mg influx systems existing in REC**

In ruminating animals, most of the required Mg is absorbed from the forestomachs by active transcellular mechanisms [19]. Therefore, unsurprisingly, REC are equipped with effective Mg influx mechanisms. These include an ion channel [21] and an Mg-Cl-cotransport [22], both of which are well characterized, at least at a functional level. However, until now the molecular identity of the ruminal Mg influx mechanisms was unknown. To our knowledge, this study shows for the first time, that TRPM7 and MagT1 are expressed in REC at both the mRNA and the protein levels. Our attempt to identify the TRPM6 transcript, which is known to be expressed in the absorptive or re-absorptive epithelia (intestine, colon and kidney; NCBI-AceView) was surprisingly unsuccessful. It could therefore be assumed that TRPM6 is not expressed in ovine REC. Both primer pairs (PP1 and PP2, figure 6A), however, have been designed and tested (figure 6A) against human TRPM6 because of the unknown sequence of the ovine TRPM6 and the expected high sequential conservation across mammals similar to those of TRPM7 and/or other TRP channels. Therefore a possibility that PP1 and PP2 do not match the ovine TRPM6 template could be also the reason. Taken together, at present, we cannot conclude whether TRPM6 is or is not expressed in REC. Identification of the ovine TRPM6 sequence and further experimentation will be necessary before making the final conclusion.

Both, MagT1 and TRPM7 have been shown to be involved in Mg uptake [6, 9, 13, 34, 54]. We therefore questioned whether the influx capacity of REC exposed to Mg deficiency or oversupply was changed. As in previous studies [5, 32], incubation of REC in Na-free media containing 5 mM Mg resulted in a marked rise in [Mg\textsuperscript{2+}], reflecting the influx of extracellular Mg. A substantial part of this Mg uptake was significantly inhibited by imipramine and Co(III)Hex. As in other cells [51, 55, 56], imipramine has been shown to be a Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger inhibitor in REC [5, 32], and Co(III)Hex is a known Mg channel blocker [13, 57]. Thus, a considerable part of the Mg influx observed under these experimental conditions resulted from the Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger working in the reverse mode [5, 32] and from channel-mediated transport pathways.

Compared with control cells, both high- and low-Mg REC are characterized by an increased Mg influx rate. In high-Mg pre-incubated REC, the larger part (51%) of their elevated influx rate resulted from facilitation of the imipramine-sensitive Na\textsuperscript{+}/Mg\textsuperscript{2+}-exchanger-related transport component. Similar imipramine effects ranging from 53% to 64% have been reported for high-Mg (5 mM) pre-incubated or high-Mg (101 mM) adapted renal epithelial cells [38, 56]. Such data are in agreement with the increased abundance and activity of the Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger found after oversupplying REC with Mg. In control and low-Mg cells however, Mg influx via reverse operating Na\textsuperscript{+}/Mg\textsuperscript{2+} exchangers amounted to 29% and 18% respectively.

In REC pre-incubated in Mg-deficient medium, the elevation of their Mg influx rate results mainly from a strong Co(III)Hex-sensitive transport component. Recently, we showed that Co(III)Hex specifically inhibits the TRPM7 channel [13]. Thus, our findings suggest that an ion-channel-mediated mechanism, most probably TRPM7, facilitates Mg influx into previously Mg-deprived REC. TRPM7 is thought to be the main regulator of cellular Mg homeostasis, and the destruction of the TRPM7 gene or its down-regulation markedly decreases Mg accumulation, reduces the basal [Mg\textsuperscript{2+}], and is followed by growth arrest and/or reduced viability of cells [4, 34, 58]. Elevated TRPM7 activity after pre-incubation of REC in Mg-deficient media should promote the replenishment of intracellular Mg stores and thus help to avoid such negative consequences of intracellular Mg deficiency.

Whether and how the REC Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger communicates with other Mg transport systems of the plasma membrane, such as TRPM7 and MagT1, or of intracellular organelles, remains unknown. He et al. [34] postulated a potential cross-talk between TRPM7 and Na\textsuperscript{+}/Mg\textsuperscript{2+} exchangers via changes of [Mg\textsuperscript{2+}]. Our own results showing a reciprocal activity of Co(III)Hex- and imipramine-sensitive influx components also point to a communication between these proteins. Such a signaling partnership between the Na\textsuperscript{+}/Mg\textsuperscript{2+} exchanger and other ion transport systems appears essential for cellular Mg homeostasis.
It has been proposed that TRPM7 has a dominant role in physiological situations in which a receptor-regulated Mg influx is required, such as the Mg uptake needed for organismal Mg homeostasis, cell growth, or neuronal functions [12]. Angiotensin II and aldosterone, for example, have been shown to regulate TRPM7 mRNA and protein content in renal and vascular smooth muscle cells [34, 59] and to stimulate angiotensin-II-mediated cell growth in the latter [34]. This would agree with the finding, that, as shown by immunoblot and flow cytometric analysis, the expression of REC TRPM7 protein does not change depending on extracellular Mg, and that its functional activity is low in non-stimulated control cells in Mg equilibrium. In contrast to high-(-29%) and low-Mg(-47%)REC, Co(III)Hex treatment reduces their Mg influx rate by only 12%. Our results agree with data from a recent in vivo study of Bruno et al. [59] who showed that Mg supplementation increases the mRNA expression of TRPM7 but has no effect on its protein content. Thus, other proteins, most probably MagT1, are presumably responsible for the background level of Mg uptake under static conditions. For MagT1, this is supported by the generally high expression and activity levels of the protein observed in our flow cytometry experiments and in functional studies with nitrendipine, an inhibitor of MagT1 channels [9].

**Conclusion**

As expected for proteins relevant to the regulation of Mg homeostasis and/or directed epithelial transport of the ion, functional activity and/or expression of REC Na+/Mg2+ exchangers, TRPM7 and MagT1 proteins are quantitatively altered by extracellular Mg deficiency or overload. Thus, we have identified these transport proteins as main components of the specific intrinsic system appropriately adapting Mg efflux and influx to Mg status. TRPM7 seems to be most important for the replenishment of intracellular Mg stores after exposure to low-Mg conditions. Independently of their Mg status, REC Na+/Mg2+ exchanger activity can be modulated by external stimuli via the 3',5'-cyclic monophosphate (cAMP)-protein kinase.

**Acknowledgments**

We express our thanks to Dr T. Viergutz (FBN Dummerstorf) for helping with the flow cytometric measurements and to Zoran Nikolic for performing the RT-PCR experiments. We gratefully acknowledge the valuable technical assistance of Renate Brose (FBN Dummerstorf) and Heike Pröhl (FBN Dummerstorf). This study was supported by a research grant from the Deutsche Forschungsgemeinschaft (Schw 642, MS) and from the Margarete-Markus foundation (Project Animal Performance and Health, MK).

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