J. Membrane Biol. 154, 217–225 (1996)

The Journal of
Membrane
Biology

Magnesium Transport Across the Basolateral Plasma Membrane of the Fish Enterocyte

M.J.C. Bijvelds[1,2], **Z.I. Kolar**[2], **S.E. Wendelaar Bonga**[1], **G. Flik**[1]

[1]Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands
[2]Department of Radiochemistry, Interfaculty Reactor Institute, Delft University of Technology, Mekelweg 15, 2629 JB Delft, The Netherlands

Received: 19 January 1996/Revised: 1 August 1996

Abstract. In tilapia (*Oreochromis mossambicus*) intestine, Mg^{2+} transport across the epithelium involves a transcellular, Na^+- and Na^+/K^+-ATPase dependent pathway. In our search for the Mg^{2+} extrusion mechanism of the basolateral compartment of the enterocyte, we could exclude Na^+/Mg^{2+} antiport or ATP-driven transport. Evidence is provided, however, that Mg^{2+} movement across the membrane is coupled to anion transport. In basolateral plasma membrane vesicles, an inwardly directed Cl^- gradient stimulated Mg^{2+} uptake (as followed with the radionuclide ^{27}Mg) twofold. As Cl^--stimulated uptake was inhibited by the detergent saponin and by the ionophore A23187, Mg^{2+} may be accumulated intravesicularly above chemical equilibrium. Valinomycin did not affect uptake, suggesting that electroneutral symport activity occurred. The involvement of anion coupled transport was further indicated by the inhibition of Mg^{2+} uptake by the stilbene derivative, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid. Kinetic analyses of the Cl^--stimulated Mg^{2+} uptake yielded a K_m (Mg^{2+}) of 6.08 ± 1.29 mmol · l^{-1} and a K_m (Cl^-) of 26.5 ± 6.5 mmol · l^{-1}, compatible with transport activity at intracellular Mg^{2+}- and Cl^--levels. We propose that Mg^{2+} absorption in the tilapia intestine involves an electrically neutral anion symport mechanism.

Key words: Magnesium — ^{27}Mg radiotracer — Mozambique tilapia — *Oreochromis mossambicus* — Basolateral plasma membrane — Mg^{2+} transport — Electrolyte metabolism

Introduction

Fresh water fish depend on magnesium absorption from the intestinal tract to meet their magnesium requirement [2, 3, 17, 31, 34, 37, 40, 42, 45]. Intestinal magnesium absorption in fish may resemble mammalian physiology where both gradient-driven paracellular transport and a transcellular component are involved [30]. Under normal feeding conditions of fish, only a minor portion of the total intestinal magnesium load will be absorbed [2, 3, 28]. The efficiency of absorption may increase, however, when the intake of magnesium via the food is low. In the teleost *Oreochromis mossambicus* (Mozambique tilapia), for instance, the fractional magnesium absorption is drastically increased when they are fed a low-magnesium diet [1]. Magnesium absorption in fish seems to be controlled by the magnesium requirement and the magnesium intake. A regulated cellular component must be involved then in transepithelial magnesium transport. Accordingly, a Na^+-dependent transepithelial magnesium flux has been demonstrated in the intestinal epithelium of tilapia, and this seems to suggest that Na^+-driven cellular magnesium transport occurs [43]. This led us to predict that a Na^+/Mg^{2+} antiport mechanism is involved in transepithelial Mg^{2+} transport.

The intracellular ionic magnesium concentration ($[Mg_i^{2+}] \leqslant 1$ mmol · l^{-1}) as well as the large transmembrane potential over the basolateral plasma membrane of enterocytes (50 to 70 mV, inside negative [19, 27], favor passive movement of Mg^{2+} into the cell, whereas Mg^{2+} extrusion must be uphill. Therefore, mucosa-to-serosa transport of Mg^{2+} could proceed by passive entry over the apical plasma membrane via an as yet unspecified transporter, followed by active extrusion across the basolateral plasma membrane. Active extrusion will also be required to maintain $[Mg_i^{2+}]$.

What drives Mg^{2+} transport over the basolateral plasma membrane of the enterocyte is not known. The energy for extrusion could be derived from hydrolysis of high-energy compounds, or energy carried by ion gradients. Mg^{2+} efflux is dependent on Na^+ antiport activity in a number of different cell types, and does in some

Correspondence to: M.J.C. Bijvelds

cases require ATP, either for activating or for energizing transport [8, 11, 16, 47]. In erythrocytes of some species, a Cl^--dependent efflux mechanism may function in parallel to Na^+ antiport activity [22–24]. The involvement of Na^+/Mg^{2+} antiport in epithelial Mg^{2+} transport remains uncertain. In mammals, Na^+/Mg^{2+} antiport activity was postulated for the Mg^{2+} reabsorption in the ascending limb of the loop of Henle [35], although recent indications suggest that Mg^{2+} absorption in the nephron proceeds predominantly passively, driven by the transepithelial potential [6, 9]. Also, the involvement of Na^+-driven Mg^{2+} transport in tubular Mg^{2+} secretion in the marine winter flounder has been suggested [38]. In trout, however, Na^+-driven Mg^{2+} transport could not be demonstrated in isolated renal brush border membrane vesicles [15].

The present study reports on Mg^{2+} transport over the basolateral plasma membrane of the intestinal epithelium of *O. mossambicus.* Mg^{2+} transport in isolated membrane vesicles was followed, using the short-lived radionuclide ^{27}Mg. The presence of ATP- or ion gradient driven Mg^{2+} transport was investigated. Evidence is presented for an electrically neutral anion coupled Mg^{2+} transport mechanism.

Materials and Methods

Mozambique tilapia, *Oreochromis mossambicus* (Peters), of both sexes were obtained from laboratory stock. Freshwater adapted fish were kept in Delft tap water ($[Mg^{2+}] = 0.3$ mmol · l^{-1}). The water temperature was 26–28°C, and the photoperiod was 12 hr of light alternating with 12 hr of darkness. Fish were fed Trouvit fish pellets (Trouw, Putten, The Netherlands) at daily rations of 1.5% of the fish total weight.

Membrane Isolation

Isolation of basolateral plasma membranes of tilapia enterocytes was carried out according to a modification of the method described by Flik *et al.* [14]. A fish, weighing approximately 200 g, was killed by spinal transection and the abdominal cavity was cut open. The 30 cm of the intestine, proximal to the stomach was excised and flushed with an ice-cold saline, containing 150 mmol · l^{-1} NaCl, 1 mmol · l^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mmol · l^{-1} 1,4-dithiothreitol (DTT), 100 U · ml^{-1} aprotinine, 0.1 mmol · l^{-1} (ethylenedinitrilo)tetraacetic (EDTA) set to pH 8.0 with 2-amino-2-hydroxymethyl-1,3-propanediol (Tris). The intestine was cut open lengthwise and spread on an ice-cooled glass plate; all further steps were performed at 0–4°C. The exposed mucosal side was rinsed with saline and the mucosal cells were scraped from the underlying tissue with a microscope slide. Scrapings were collected in sucrose buffer, containing 250 mmol · l^{-1} sucrose, 10 mmol · l^{-1} HEPES, 1 mmol · l^{-1} DTT, 100 U · ml^{-1} aprotinine, set to pH 7.4 with Tris, and disrupted with a dounce homogenizer equipped with a loosely fitting pestle (30 strokes). The homogenate was centrifuged for 10 min at 1,400 × *g* (Jouan CR3000, E4 rotor, 2700 rpm) to remove nuclei and cellular debris. The supernatant was centrifuged for 30 min at 186,000 × *g* (Beckman L7-55, 70.1 Ti rotor, 45,000 rpm), yielding a membrane pellet consisting of a brownish part well-fixed to the wall of the tube (containing a mitochondrial membrane fraction) and a fluffy layer on top. The fluffy part of the pellet was removed by gentle stirring, resuspended in 18 ml of sucrose buffer, and homogenized in a dounce homogenizer (80 strokes). The homogenate was brought to 1.36 mol · l^{-1} (39% wt/wt) sucrose by mixing with 22.5 ml 2.25 mmol · l^{-1} sucrose, 10 mmol · l^{-1} HEPES/Tris (pH 7.4). The resulting suspension was equally divided over four centrifuge tubes, overlaid with 2 ml of sucrose buffer and centrifuged isopycnically for 2 hr at 154,000 × *g* (Beckman L7-55, SW41 Ti rotor, 30,000 rpm). The membranes at the interface of the sucrose solutions were collected in 10 ml isotonic buffer, containing the basic ingredients of the assay medium (*see* the section Mg^{2+} Transport in Plasma Membrane Vesicle Preparations for details on the composition of the assay medium). The membranes were pelletized by centrifugation, 30 min at 186,000 × *g* (Beckman L7-55, 70.1 Ti rotor, 45,000 rpm), rinsed with isotonic buffer, and resuspended by 20 passages through a 23-Gauge needle in 0.35 ml isotonic buffer. Membrane preparations contained approximately 2.5 mg · ml^{-1} protein and were used on the day of isolation without being frozen. Protein concentration was determined with a commercial reagent kit (Biorad), with bovine serum albumin as reference.

Enzyme Assays

Na^+/K^+-ATPase activity was assayed as a marker enzyme for basolateral plasma membranes [33]. Enzyme activity was assayed after treatment with detergent, 0.2 mg · ml^{-1} saponin (10 min at 25°C), at a protein concentration of 1 mg · ml^{-1}, to unmask enzyme activity that was latent due to membrane resealing. The basolateral plasma membrane preparation was 8.7 ± 1.5 ($n = 6$) fold enriched in Na^+/K^+-ATPase activity, when compared to the initial tissue homogenate. The specific activity of the enzyme (P_i release) was 143 ± 25 μmol · h^{-1} mg^{-1} protein ($n = 6$), a value comparable to the Na^+/K^+-ATPase activity reported earlier for a basolateral membrane preparation of tilapia enterocytes [14]. The purification factor for the enzyme sucrase was 0.56 ± 0.10 ($n = 6$), indicating that the preparation was not enriched in apical plasma membranes [4].

Membrane orientation was determined as described previously [14]. The percentage inside-out oriented vesicles (IOV) was determined on the basis of acetylcholine esterase activity, using 0.2 mg · ml^{-1} saponin (10 min at 25°C) to unmask latent enzyme activity. Determination of the percentage rightside-out oriented vesicles (ROV) was based on the specific trypsin sensitivity of the cytosol-oriented part of the Na^+/K^+-ATPase. Trypsin (T-8003; Sigma, St. Louis, MO) was used at 4500 units per mg membrane protein, for 30 min at 25°C. After quenching of trypsin activity with 25 mg · ml^{-1} soybean trypsin inhibitor (T-9003; Sigma, St. Louis, MO), trypsin-insensitive Na^+/K^+/ATPase activity (representing the ROV fraction) was unmasked by treatment with saponin. In controls, trypsin inhibitor was added before the addition of trypsin to assess total Na^+/K^+-ATPase activity. The membrane configuration was 15 ± 5% IOV, 30 ± 8% ROV and 55 ± 7% leaky membrane fragments ($n = 6$). The presence of inside-out oriented basolateral plasma membrane vesicles was further demonstrated by the assay of ATP- and Na^+-dependent Ca^{2+}-uptake.

Production of ^{27}Mg

MgO, isotopically enriched in ^{26}Mg to 97.1% (Medgenics Group, Ratingen, Germany), was dissolved in diluted ''Suprapur'' acetic acid (Merck, Darmstadt, Germany), resulting in a 50 mmol · l^{-1} $Mg(CH_3COO)_2$ solution. ^{27}Mg (half-life 9.46 min) was produced by irradiation of this solution in a thermal neutron flux of $4 \cdot 10^{16}$ $m^{-2} \cdot sec^{-1}$ for 10 min in the Interfaculty Reactor Institute nuclear

reactor. The specific activity of the ^{27}Mg preparation directly after irradiation was approximately 35 GBq · mol^{-1}.

Mg^{2+} Transport in Plasma Membrane Vesicle Preparations

Transport of Mg^{2+} was assayed by means of a rapid filtration technique [29].

Na^+/Mg^{2+} antiport was assayed by adding 10 μl of membrane vesicle preparation in 150 mmol · l^{-1} NaCl, 20 mmol · l^{-1} HEPES/Tris (pH 7.4) to 140 μl of assay medium, yielding the following composition: 140 mmol · l^{-1} KCl, 10 mmol · l^{-1} NaCl, 0.1 mmol · l^{-1} ethyleneglycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), 120 nmol · l^{-1} free Ca^{2+} and 20 mmol · l^{-1} HEPES/Tris (pH 7.4). [^{27}Mg]-Mg(CH_3COO)$_2$ was added to the assay medium at a final concentration of 2 mmol · l^{-1}. In some cases 1 mmol · l^{-1} ATP was added. Na^+-dependent Mg^{2+} uptake was determined as the difference between uptake in K^+- or Na^+ medium, using NaCl to replace KCl isosmotically. When Na^+/Ca^{2+} antiport was assayed in parallel, [Ca^{2+}] was set to 1 μmol · l^{-1}, a trace quantity of [^{45}Ca]-$CaCl_2$ (NEN/Du Pont, ′s, Hertogenbosch, The Netherlands) was added to the assay medium and [^{27}Mg]-Mg(CH_3COO)$_2$ was replaced by $MgCl_2$.

Anion-dependent Mg^{2+} transport was assayed by adding 10 μl of membrane vesicle preparation in 126 mmol · l^{-1} sodium isethionate, 20 mmol · l^{-1} sodium gluconate or potassium gluconate, 4.3 mmol · l^{-1} $NaCH_3COO$ and 20 mmol · l^{-1} HEPES/Tris (pH 7.4) to 140 μl of assay medium, yielding the following composition: 120 mmol · l^{-1} NaCl (or 120 mmol · l^{-1} $NaNO_3$ or sodium gluconate, or 60 mmol · l^{-1} Na_2SO_4), 8.4 mmol · l^{-1} sodium isethionate, 0.3 mmol · l^{-1} $NaCH_3COO$, 0.1 mmol · l^{-1} EGTA, 120 nmol · l^{-1} free Ca^{2+} and 20 mmol · l^{-1} HEPES/Tris, pH 7.4. [^{27}Mg]-Mg(CH_3COO)$_2$ was added to the assay medium at a final concentration of 0.5 to 2 mmol · l^{-1}. Mg^{2+} (gluconic acid salt) was added to obtain calculated free Mg^{2+} concentrations ranging from 0.45 to 9.07 mmol · l^{-1}. The ionic strength of the medium was adjusted to 0.157 M by addition of sodium gluconate or potassium gluconate. Anion-dependent uptake was determined as the difference between Mg^{2+} uptake in the presence or absence of the respective anion, using isethionate to replace it isosmotically. In some cases N-methyl-D-glucamine ($NMDG^+$, Cl^- or gluconic acid salt) was used to replace monovalent cations.

ATP-dependent Mg^{2+} uptake was assayed by diluting (15×) membrane vesicle preparations loaded with 150 mmol · l^{-1} KCl, 20 mmol · l^{-1} HEPES/Tris (pH 7.4), in assay medium containing 150 mmol · l^{-1} KCl, 0.1 mmol · l^{-1} EGTA, 120 mmol · l^{-1} free Ca^{2+}, 1 mmol · l^{-1} ATP and 20 mmol · l^{-1} HEPES/Tris, pH 7.4 [^{27}Mg]-Mg(CH_3COO)$_2$ was added to the assay medium at a final concentration of 2 mmol · l^{-1}. ATP-dependent uptake was determined as the difference between Mg^{2+} uptake in the presence or absence of ATP. In the absence of ATP, 0.97 mmol · l^{-1} trans-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA) was added to replace ATP as a Mg^{2+} chelator, thus keeping total and free Mg^{2+} concentrations equal under both conditions. In some instances ATP was replaced by 1 mmol · l^{-1} adenosine 5′-O-(3-thiotriphosphate) (ATP-γ-S). When Ca^{2+} pump activity was assayed in parallel, [Ca^{2+}] was set to 1 μmol · l^{-1}, a trace quantity of [^{45}Ca]-$CaCl_2$ was added to the assay medium and [^{27}Mg]-Mg(CH_3COO)$_2$ was replaced by $MgCl_2$.

Equilibrium Mg^{2+} uptake was assayed by diluting (15×) membrane vesicle preparations loaded with 150 mmol · l^{-1} potassium gluconate, 20 mmol · l^{-1} HEPES/Tris (pH 7.4) in assay medium containing 120 to 250 mmol · l^{-1} potassium gluconate and 20 mmol · l^{-1} HEPES/Tris (pH 7.4). [^{27}Mg]-Mg(CH_3COO)$_2$ was added to obtain a calculated free [Mg^{2+}] of 0.5 mmol · l^{-1}.

Incubations were carried out at a membrane protein concentration of 0.12 to 0.27 mg · ml^{-1}, at 28°C in media of 0.157 M ionic strength. The radioactive concentration was 15 to 60 kBq · ml^{-1} of ^{27}Mg or approximately 300 kBq · ml^{-1} of ^{45}Ca. Free Ca^{2+} and free Mg^{2+} concentrations were calculated according to Schoenmakers et al. [39], taking into account the metal ion complexation with EGTA, CDTA, ATP and gluconate [41]. A23187 stock was dissolved in dimethyl sulfoxide (DMSO) and added to the assay medium at 5 μmol · $^{-1}$. Valinomycin stock was dissolved in ethanol. Valinomycin (10 μmol · l^{-1}) and saponin (0.02%, w/v) were added to membrane preparations kept on ice, 10 min before Mg^{2+} transport was assayed. Concentration of solvents in the assay medium did not exceed 0.2% (v/v) and solvent treatment was used as control. Furosemide, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid (DIDS) and chlorothiazide were added to the assay medium just prior to experimentation at a final concentration of 0.5 mmol · l^{-1}.

The reaction was quenched by addition of 1-ml ice-cold stop buffer (150 mmol · l^{-1} sodium gluconate, 1.5 mmol · l^{-1} $MgCl_2$, 0.1 mmol · l^{-1} $LaCl_3$, and 20 mmol · l^{-1} HEPES/Tris at pH 7.4) to 150 μl incubate. A volume of 1 ml, equivalent to 15 to 35 μg membrane protein, was filtered on 0.45 μm ME25 membrane filters (Schleicher & Schuell, Dassel, Germany) at a reduced pressure of 75 kPa. Filters were rinsed twice with 2 ml of stop buffer and transferred to 10 ml scintillation cocktail. Radioisotope specific activity was determined by counting the radioactivity in 0.100 ml of the remaining ''quenched reaction'' suspension. Radioactivity was determined on the basis of the β^- emission of ^{27}Mg and ^{45}Ca in a Tri-Carb 2750TR/LL liquid scintillation analyzer (Packard Instrument, Meridan, CT). Counting rates were corrected for background and radioactive decay.

Calculations and Statistics

Kinetic analysis of $^{27}Mg^{2+}$ uptake was performed using a nonlinear regression analysis programme [7]. Values are expressed as mean ± SD, unless stated otherwise. Statistical significance of differences between means was assessed using the two-tailed Student's *t* test and accepted at $P < 0.05$.

Results

Equilibrium Mg^{2+} Uptake

Basal, unstimulated Mg^{2+} uptake plateaued after approximately 3 min and thus Mg^{2+} uptake after 5-min incubation reflects equilibrium uptake. The dependence of equilibrium Mg^{2+} uptake on the vesicular volume (Fig. 1) indicates uptake in the osmotic space. Linear regression yielded the Y-axis intercept which signifies vesicular volume independent membrane-bound Mg^{2+}. At an osmolarity of 300 mosmol · kg^{-1} H_2O the Mg^{2+} uptake in the osmotic space amounted to 2.45 ± 1.07 nmol · mg^{-1} protein. On the basis of a free [Mg^{2+}] = 0.5 mmol · l^{-1}, assuming that at equilibrium the intravesicular [Mg^{2+}] equals the extravesicular [Mg^{2+}], we calculate a Mg^{2+} distribution space of 4.9 ± 2.1 μl · mg^{-1}. Similar experiments in which sucrose was used to manipulate osmolarity revealed that volume independent binding is enhanced in non-ionic media. Therefore, further assays were carried out in ionic media of constant ionic

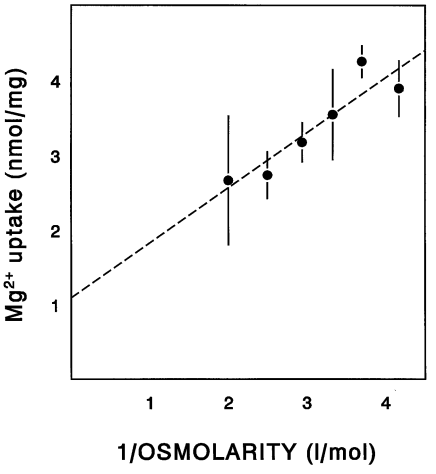

Fig. 1. Equilibrium $^{27}Mg^{2+}$ uptake in plasma membrane vesicles. Preparations were incubated for 5 min in ionic media at the osmolarity indicated, in the presence of 0.5 mmol · l^{-1} Mg^{2+}. Means ± SEM of three preparations are given.

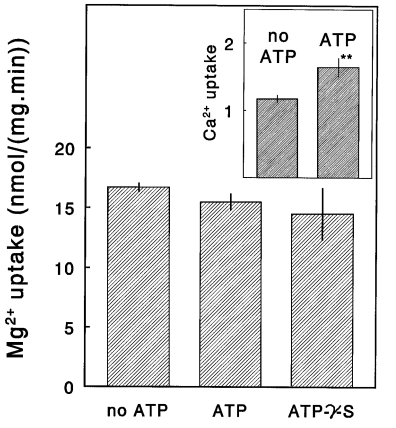

Fig. 2. ATP-driven $^{27}Mg^{2+}$ uptake in plasma membrane vesicles. Preparations were incubated for 1 min, in the presence of 1.8 mmol · l^{-1} Mg^{2+}. ATP and ATP-γ-S were added at 1 mmol · l^{-1}. Means ± SD of five preparations are given. The inset shows the ATP-driven $^{45}Ca^{2+}$ uptake (in nmol · min^{-1} · mg^{-1} protein), measured in parallel experiments (**$P < 0.01$; n = 3).

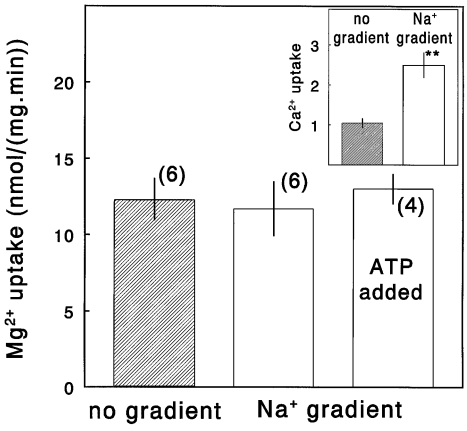

Fig. 3. Na^+-driven $^{27}Mg^{2+}$ uptake in plasma membrane vesicles. Preparations were incubated for 1 min, in the presence of 1.8 mmol · l^{-1} Mg^{2+}. A Na^+ gradient (open bars) was created by dilution of Na^+-loaded vesicles in K^+ medium. Where indicated, ATP was added at 1 mmol · l^{-1}. The number of experiments is indicated between brackets. The inset shows the Na^+-driven $^{45}Ca^{2+}$ uptake (in nmol · min^{-1} · mg^{-1} protein), measured in parallel experiments (**$P < 0.01$; n = 3).

strength, to ensure that the membrane-bound magnesium fraction was of a comparable magnitude in all experiments. This allowed a direct statistical comparison of Mg^{2+} uptake under the various conditions tested, without prior correction of the data for the membrane-bound Mg^{2+} fraction. Further data are presented accordingly without correction for the membrane-bound Mg^{2+} fraction.

ATP-Dependent Mg^{2+} Transport

Figure 2 shows the ATP-independence of Mg^{2+} uptake in plasma membrane vesicles. ATP at 1 mmol · l^{-1} did not stimulate Mg^{2+} uptake. Also, replacement of ATP by its nonhydrolyzable analogue ATP-γ-S did not affect Mg^{2+} uptake significantly. The inset of Fig. 2 shows that in parallel experiments ATP-dependent Ca^{2+} pump activity could be demonstrated.

Na^+/Mg^{2+} Antiport Activity

Na^+/Mg^{2+} antiport activity was assayed in the presence of an outwardly directed Na^+-gradient to drive Mg^{2+} into the vesicular space. Mg^{2+} uptake, assayed at 1 min, was not stimulated by the outwardly directed Na^+ gradient, and ATP had no effect on this process (Fig. 3). The inset of Fig. 3 shows that in parallel experiments Na^+/Ca^{2+} antiport activity could be demonstrated.

Evidence for Anion-dependent Mg^{2+} Transport

Mg^{2+} uptake was stimulated by inwardly directed gradients (parallel to the Mg^{2+} movement) of membrane permeable anions like Cl^-, SO_4^{2-} and NO_3^-, but not by the membrane impermeable anion gluconate (Fig. 4). Voltage clamping of the vesicular membrane with valinomycin, at 20 mmol · l^{-1} K^+ inside and outside, did not affect the anion-dependent Mg^{2+} uptake significantly: Cl^--stimulated Mg^{2+} uptake in the presence of valinomycin was 96 ± 15% of solvent control (n = 5). The stimulation of Mg^{2+} uptake in the presence of an inwardly directed Cl^- gradient was independent of the concurrent presence of inwardly directed gradients of either Na^+ or K^+ (Fig. 5). A small but significant decrease in Mg^{2+}

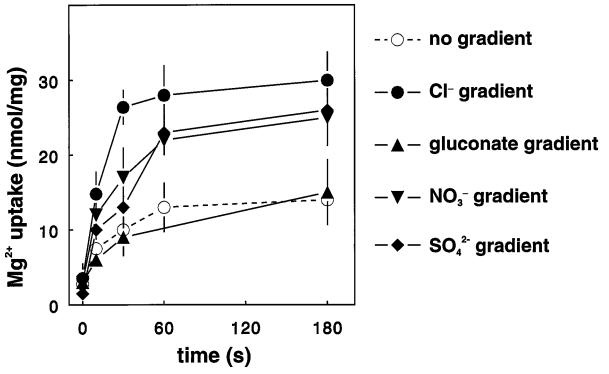

Fig. 4. Time-dependence of Mg^{2+} uptake in plasma membrane vesicles in the presence of inwardly directed gradients of Cl^-, SO_4^{2-}, NO_3^-, and gluconate. The broken line indicates the Mg^{2-} uptake in the absence of ion gradients. Preparations were incubated during the time indicated, in the presence of 1.8 mmol · l^{-1} Mg^{2+}. Data are means of four preparations.

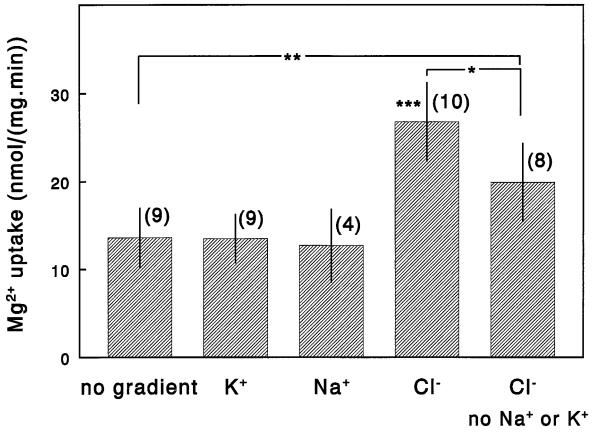

Fig. 5. Effect of inwardly directed Cl^-, Na^+- and K^+ uptake in plasma membrane vesicles. Preparations were incubated for 1 min, in the presence of 1.8 mmol · l^{-1} Mg^{2+} and gradients of the ions indicated. Na^+ and K^+ were replaced by equimolar $NMDG^+$. The effects of ion gradients were statistically compared to the ''no gradient'' control. The number of experiments is indicated between brackets. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

uptake was observed when all Na^+ and K^+ in the assay medium was replaced by $NMDG^+$.

Cl^- dependent Mg^{2+} uptake was not inhibited by furosemide or chlorothiazide, but DIDS abolished Cl^- stimulation of Mg^{2+} uptake (Fig. 6). The Cl^--independent Mg^{2+} uptake in the presence of DIDS was 95 ± 5% (n = 3) of the control and, therefore, DIDS did not affect the basal, unstimulated Mg^{2+} uptake. Permeabilization of vesicles by saponin-treatment prevented Cl^--dependent Mg^{2+} uptake, and addition of Mg^{2+}-ionophore A23187 caused a small but significant decrease in Mg^{2+} uptake (Fig. 7).

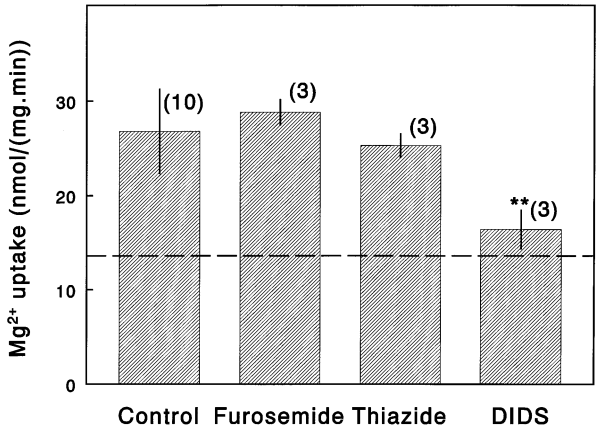

Fig. 6. Effect of diuretics on Cl^--stimulated $^{27}Mg^{2+}$ uptake in plasma membrane vesicles. Preparations were incubated for 1 min, in the presence of 1.8 mmol · l^{-1} Mg^{2+} and 0.5 mmol · l^{-1} of the substances indicated. Broken line indicates the level of Cl^--independent Mg^{2+} uptake (*see* Fig. 5). The number of experiments is indicated between brackets. **$P < 0.01$

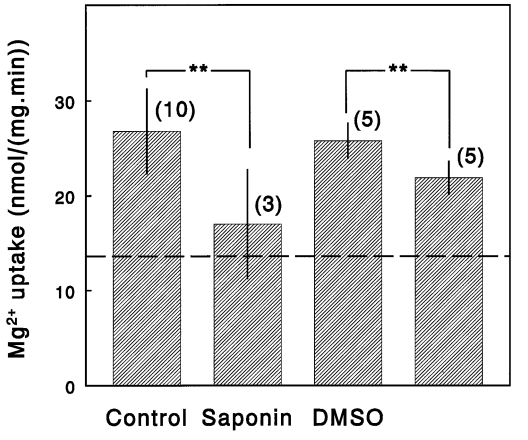

Fig. 7. Effect of membrane permeabilization on Cl^--stimulated $^{27}Mg^{2+}$ uptake in plasma membrane vesicles. Preparations were incubated for 1 min, in the presence of 1.8 mmol · l^{-1} Mg^{2+}. Saponin was added at 0.02% (w/v) and A23187 was added at 5 μmol · l^{-1}. DMSO (0.2% v/v) was used as solvent control. Dashed line indicates the level of Cl^--independent Mg^{2+} uptake (*see* Fig. 5). The number of experiments is indicated between brackets. **$P < 0.01$.

Kinetics of Cl^--dependent Mg^{2+} Uptake

Initial rates of Mg^{2+} uptake were estimated from 15 sec incubations (Fig. 4) and Cl^--dependent Mg^{2+} uptake was determined as the difference between Mg^{2+} uptake in the presence or absence of a Cl^- gradient. The dependence of Mg^{2+} uptake on [Mg^{2+}] followed single Michaelis-Menten kinetics. A K_m of 6.08 ± 1.29 mmol · l^{-1} and a V_m of 32.0 ± 4.4 nmol · 15 sec^{-1} mg^{-1} protein were derived (Fig. 8*a*). [Cl^-]-dependence of Mg^{2+} uptake

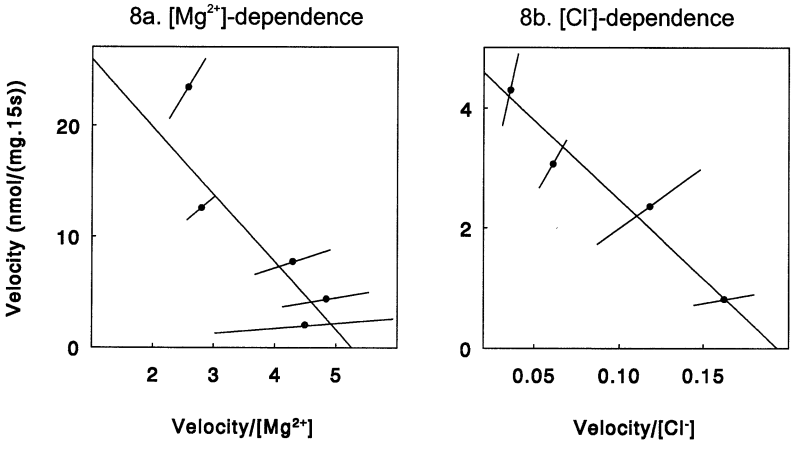

Fig. 8. $[Mg^{2+}]$- and $[Cl^-]$-dependence of Cl^--stimulated $^{27}Mg^{2+}$ uptake in plasma membrane vesicles. Initial rates of uptake were determined in 15-sec incubations and values were corrected for Cl^--independent uptake. Data of three to six preparations were fitted to the Michaelis-Menten equation, yielding the kinetic parameters indicated in the text. The graphs show an Eadie-Hofstee transformation of the data.

yielded a K_m (Cl^-) of 26.5 ± 6.5 mmol · l^{-1} (Fig. 8*b*). V_m (Mg^{2+}), assayed at 1.8 mmol · l^{-1} Mg^{2+}, was 5.12 ± 0.42 nmol · 15 sec^{-1} · mg^{-1} protein.

Discussion

A novel Mg^{2+} transporter is demonstrated in a vertebrate intestinal cell. Mg^{2+} transport over the basolateral membrane of tilapia enterocytes was ATP- and Na^+-*in*dependent, but transmembrane gradients of membrane permeable anions stimulated transport. Electrodiffusive transport was not indicated as voltage clamping of the vesicular membrane did not affect the Mg^{2+} uptake. We suggest that Mg^{2+} transport proceeded in an electrically silent manner, as no buildup of a membrane potential during Mg^{2+} uptake was indicated. These data are compatible with a Mg^{2+} anion symport mechanism, where the anion drives electroneutral transmembrane Mg^{2+} transport. *In vivo,* this mechanism may provide a Mg^{2+} extrusion mechanism that is involved in maintaining $[Mg_i^{2+}]$ and in transepithelial Mg^{2+} transport.

Equilibrium Mg^{2+} Uptake

Our data indicate that the plasma membrane vesicles used in this study are osmotically active and that Mg^{2+} is transported into the osmotic space of the vesicle. The calculated Mg^{2+} distribution space of 4.9 ± 2.1 μl · mg^{-1} is comparable to the mannitol space of 3 μm · mg^{-1} reported for a similar plasma membrane preparation [14].

Na^+- and ATP-Coupled Mg^{2+} Transport

Na^+/Mg^{2+} antiport activity is involved in regulation of $[Mg_i^{2+}]$ in a range of cell types, like erythrocytes, hepatocytes and neuronal cells [8, 13, 20, 26]. Besides being dependent on extracellular Na^+, the Mg^{2+} efflux mechanism present in erythrocytes of some species is also dependent on ATP [8, 16, 47]. So far, however, no direct evidence has been presented for a Na^+- or ATP-coupled Mg^{2+} transporter in ion transporting epithelia. In our experiments, Mg^{2+} uptake in plasma membrane vesicles of tilapia intestine was not affected by ATP, nor by its nonhydrolyzable analogue ATP-γ-S, and this excludes the possibility that Mg^{2+} transport proceeds through an ATPase ion pump, or that ATP is required for activation of Mg^{2+} transport. We could neither demonstrate Na^+-driven Mg^{2+} transport in this membrane vesicle preparation of tilapia intestine, in both the absence and presence of ATP. One may argue that an unknown factor required for the proper functioning of an antiport mechanism (like a protein kinase) is lost during the membrane isolation procedure. However, Na^+/Mg^{2+} antiport activity has also been demonstrated in resealed ghosts of erythrocytes, which suggests that the whole cell configuration is not a prerequisite for its functioning [16]. Furthermore, the absence of Na^+-driven Mg^{2+} transport can not be ascribed to a rapid dissipation of the Na^+ gradient: it was demonstrated that, in the presence of extravesicular Mg^{2+}, a substantial Na^+ gradient can be maintained over the vesicular membrane that is capable of driving Ca^{2+} uptake. We tentatively conclude, therefore, that no Na^+- or ATP-coupled Mg^{2+} transport mechanism is present in the basolateral plasma membrane of tilapia enterocytes.

Anion-Coupled Mg^{2+} Transport

Our data indicate a clear dependence of Mg^{2+} uptake in plasma membrane vesicles on an inwardly directed gradient of membrane permeable anions like Cl^-, SO_4^{2-} and NO_3^-. Diffusion of anions into the vesicular space may create a transmembrane potential favoring Mg^{2+} influx. However, voltage clamping of the membrane potential did not prevent the stimulation of Mg^{2+} uptake by a Cl^- gradient, arguing against voltage-driven transport. Furthermore, the dependence of Mg^{2+} transport on both extravesicular $[Mg^{2+}]$ and $[Cl^-]$ displayed Michaelis-

Menten kinetics, indicating a carrier- or channel-mediated, nondiffusive, transport principle. Anion-dependent Mg^{2+} transport has been described for Yoshida ascites tumor cells [25], hepatocytes [21] and erythrocytes [22, 23]. In Yoshida ascites tumor cells and in rat hepatocytes Mg^{2+} influx displayed saturation kinetics and kinetic analysis suggested electroneutral Mg^{2+}/anion symport activity. Mg^{2+} influx in rat hepatocytes was dependent on inorganic phosphate, but could also be accompanied by Cl^- and HCO_3^- symport [21]. In Yoshida ascites tumor cells Mg^{2+} influx was driven by HCO_3^- symport, although other anions may also be involved [25].

The powerful inhibition of Cl^--dependent Mg^{2+} uptake by the stilbene derivative DIDS further supports the involvement of an anion transport mechanism in Mg^{2+} transport. Stilbene-sensitive anion transporters located in the basolateral membranes of the intestine have been described for rat, rabbit and the urodele *Amphiuma* [18, 32, 46]. Similar to the transport mechanism described in the present study, some of these stilbene-sensitive transporters accept a broad range of inorganic anions. In rabbit ileum, for instance, the antiporter involved in active SO_4^{2-} absorption, exchanges SO_4^{2-} for either Cl^-, Br^-, I^-, NO_3^- or SO_4^{2-}, but not gluconate [32]. Stilbene-sensitive Mg^{2+} transport has been described for mammalian and avian erythrocytes [22, 23]. In erythrocytes, Cl^- symport prevents the buildup of an inhibitory potential difference across the plasma membrane which would result from the extrusion of net positive charge [24]. In the present study, the insensitivity of Cl^--coupled Mg^{2+} transport to clamping of the membrane potential with valinomycin suggests that transport, similar as in the erythrocyte system, occurs in an electroneutral mode, i.e., with a coupling ratio of 1 Mg^{2+}/2 Cl^-. However, the kinetic analysis of Cl^--coupled Mg^{2+} transport did not reveal the presence of two binding sites for Cl^- (Hill coefficient is 0.82 ± 0.34) which would corroborate this coupling ratio. Therefore, conclusive evidence for the stoichiometry of the process can not be derived from the present data.

The Mg^{2+} uptake in the presence of a Cl^- gradient of approximately 28 nmol · mg^{-1} protein at 1 min was considerably higher than predicted equilibrium values: the Mg^{2+} uptake after membrane permeabilization by saponin, which will facilitate equilibration of Mg^{2+} and Cl^- across the vesicular membrane, of 17.0 ± 5.8 nmol mg^{-1} protein, and the calculated equilibrium Mg^{2+} uptake of 12.4 nmol · mg^{-1} protein, as calculated from the Mg^{2+}-distribution space (4.9 ± 2.1 µl · mg^{-1} protein), the medium Mg^{2+} concentration (1.8 mmol · l^{-1}), and taking into account an estimated, vesicular space-independent, membrane-bound fraction of 4 nmol · mg^{-1} protein (extrapolated from Fig. 1). As our experimental setup does not allow a distinction to be made between Mg^{2+} uptake in IOVs or ROVs, this calculation assumes that Mg^{2+} is accumulated in both, i.e., that the Mg^{2+} transporter behaves symmetrically. In support of this assumption, kinetic analysis of the Mg^{2+} uptake indicated a uniform mechanism, suggesting that uptake in IOVs and ROVs proceeds through an identical mechanism. We conclude that imposing a Cl^- gradient can drive Mg^{2+} accumulation above chemical equilibrium. Uphill Mg^{2+} transport was further indicated by the inhibition of uptake by the Mg^{2+} ionophore A23187.

Whether Cl^- can drive Mg^{2+} extrusion *in vivo* will depend on the energy stored in the Cl^- gradient across the serosal membrane of the enterocyte and the coupling ratio of Mg^{2+}/Cl^- symport. It is generally accepted that Cl^- is kept above electrochemical equilibrium in fish intestinal cells. Accumulation is driven by Na^+-coupled Cl^- influx via the apical membrane [12, 36], although the presence of a conductive pathway for Cl^- entry has also been suggested [5]. This secondary active transport at the apical pole, ultimately driven by the Na^+ pump, provides the driving-force for Cl^- absorption across the basolateral membrane, putatively through K^+/Cl^- symport and/or through a Cl^- conductive pathway [10, 27, 48]. The driving force for Cl^- extrusion reported for goldfish and winter flounder are 27 and 25 mV, respectively [10, 48]. Proceeding from a membrane potential of −60 mV [19, 27], a [Mg_i^{2+}] below 1 mmol · l^{-1} and a plasma [Mg^{2+}] of 0.51 mmol · l^{-1} [44], one can calculate that a driving force of at least 51 mV is required to drive Mg^{2+} extrusion. Therefore, symport of 2 Cl^- per Mg^{2+} may suffice to drive Mg^{2+} extrusion.

Cl^- coupled Mg^{2+} transport was partially dependent on the presence of K^+ or Na^+, suggesting that these cations may activate or drive Mg^{2+} transport. For rat hepatocytes it was proposed that Na^+ symport provides the driving force for anion-dependent Mg^{2+} influx [21]. This system was insensitive to bumetanide, indicating that the Na(K)Cl-symporter of the so-called NKCC-family was not involved in Mg^{2+} transport. A similar conclusion was drawn for the Mg^{2+} influx mechanism of Yoshida ascites tumor cells [25]. In line with these studies, the Mg^{2+} transport system of the tilapia intestine was insensitive to furosemide and chlorothiazide. From this we conclude that Mg^{2+} transport does not proceed via a NKCC-family transporter. However, we cannot exclude a cation-dependent Na^+ transport mechanism that is insensitive to these loop diuretrics, like the Mg^{2+} transport system of rat hepatocytes [21]. Another explanation for the observed inhibition of Mg^{2+} transport may be that complete Na^+ and K^+ replacement by the inert cation $NMDG^+$ inhibits ion transport by affecting the integrity of the vesicular membrane.

For tilapia intestine, it was shown that the mucosa-to-serosa Mg^{2+}-flux is dependent on extracellular Na^+ and can be inhibited by ouabain [43]. From our data, we

conclude that this apparent dependence of Mg^{2+} transport on Na^+ is indirect as no Na^+-driven Mg^+ transport was indicated, but may reflect a dependence on Na^+-driven Cl^- accumulation. Mg^{2+} transport may thus be coupled to stilbene-sensitive downhill Cl^- extrusion across the basolateral membrane. A similar stilbene-sensitive, Cl^--dependent, Mg^{2+} efflux mechanism has been described for avian and mammalian erythrocytes [22, 23]. With the demonstration of such a carrier in fish, it seems of wider occurrence in vertebrates.

The authors gratefully acknowledge Ms. C. Zegers and Mr. W. den-Hollander for technical assistance and Professor J.J.M. de Goeij for his critical comments during the preparation of the manuscript.

References

1. Bijvelds, M.J.C., Flik, G., Kolar, Z.I., Wendelaar Bonga, S.E. 1996. Uptake, distribution and excretion of magnesium in *Oreochromis mossambicus:* dependence on magnesium in diet and water. *Fish Physiol. Biochim.* (*in press*)
2. Dabrowska, H., Meyer-Burgdorff, K.H., Günther, K.D. 1989. Interaction between dietary protein and magnesium level in tilapia (*Oreochromis niloticus*). *Aquaculture* **76:**277–291
3. Dabrowska, H., Meyer-Burgdorff, K.H., Günther, K.D. 1991. Magnesium status in freshwater fish, common carp (*Cyprinus carpio,* L.) and the dietary protein-magnesium interaction. *Fish Physiol. Biochem.* **9:**165–172
4. Dahlavist, A. 1964. Method for assay of intestinal disaccharides. *Analyt. Biochem.* **7:**18–25
5. De Giorgi, A., Carmineo, L., Corcelli, A. 1992. Mechanism of Cl^- transport in eel intestinal brush-border membrane vesicles. *Pfluegers Arch.* **420:**551–558
6. Derouffignac, C., Quamme, G. 1994. Renal magnesium handling and its hormonal control. *Physiol. Rev.* **74:**305–322
7. Dharmamba, M., Bornancin, M., Maetz, J. 1975. Environmental salinity and sodium and chloride exchange across the gill of *Tilapia mossambica. J. Physiol.* **70:**627–636
8. DiPolo, R., Beaugé, L. 1988. An ATP-dependent Na^+/Mg^{2+} countertransport is the only mechanism for Mg^{2+} extrusion in squid axons. *Biochim. Biophys. Acta* **946:**424–428
9. Distefano, A., Roinel, N., De Rouffignac, C., Wittner, M. 1993. Transepithelial Ca^{2+} and Mg^{2+} transport in the cortical thick ascending limb of henles loop of the mouse is a voltage-dependent process. *Renal Physiol. Biochem.* **16:**157–166
10. Duffey, M.E., Thompson, S.M., Frizzell, R.A., Schultz, S.G. 1979. Intracellular chloride activities and active chloride absorption in the intestinal epithelium of the winter flounder. *J. Membrane Biol.* **50:**331–341
11. Féray, J.-C., Garay, R. 1986. An Na^+-stimulated Mg^{2+}-transport system in human red blood cells. *Biochim. Biophys. Acta* **856:**76–84
12. Field, M., Karnaky, K.J., Jr., Smith, P.L., Bolton, J.E., Kinter, W.K. 1978. Ion transport across the isolated intestinal mucosa of the winter flounder, *Pseudopleuronectes americanus.* I. Functional and structural properties of cellular and paracellular pathways for Na and Cl. *J. Membrane Biol.* **41:**265–293
13. Flatman, P.W. 1992. Magnesium and transport in red cells. *J. Trace Elem. Electrolytes Health Dis.* **6:**1–5
14. Flik, G., Schoenmakers, T.J.M., Groot, J.A., Van, Os, C.H., Wendelaar Bonga, S.E. 1990. Calcium absorption by fish intestine: the involvement of ATP- and sodium-dependent calcium extrusion mechanisms. *J. Membrane Biol.* **113:**13–22
15. Freire, C.A., Kinne, R.K.H., Kinne-Saffran, E., Beyenbach, K.W. 1996. Electrodiffusive transport of Mg across renal membrane vesicles of the rainbow trout *Oncorhynchus mykiss. Am. J. Physiol.* **270:**F739–F748
16. Frenkel, E.J., Graziani, M., Schatzmann, H.J. 1989. ATP requirement of the sodium-dependent magnesium extrusion from human red blood cells. *J Physiol.* **414:**385–397
17. Gatlin, D.M., III, Robinson, E.H., Poe, W.E., Wilson, R.P. 1982. Magnesium requirement of fingerling channel catfish and signs of magnesium deficiency. *J. Nutr.* **112:**1182–1187
18. Grinstein, S., Turner, R.J., Silverman, M., Rothstein, A. 1980. Inorganic anion transport in kidney and intestinal brush border and basolateral membranes. *Am. J. Physiol.* **238:**F452–F460
19. Groot, J.A., Albus, H., Bakker, R., Dekker, K. 1983. Changes in sugar transport and in electrophysiological characteristics of intestinal preparations of temperature-acclimated goldfish (*Carassius auratus* L.). *J. Comp. Physiol.* **151:**163–170
20. Günther, T., Höllriegl, V. 1993. Characterization of Na^+-dependent Mg^{2+} efflux from isolated hepatocytes. *Magnesium Bull.* **15:**121–123
21. Günther, T., Höllriegl, V. 1993. Na^+- and anion-dependent Mg^{2+} influx in isolated hepatocytes. *Biochim. Biophys. Acta* **1149;**49–54
22. Günther, T., Vormann, J. 1989. Characterization of Mg^{2+} efflux from human, rat and chiken erythrocytes. *FEBS Lett.* **250:**633–637
23. Günther, T., Vormann, J. 1989. Na^+-independent Mg^{2+} efflux from Mg^{2+}-loaded human erythrocytes. *FEBS Lett.* **247:**181–184
24. Günther, T., Vormann, J. 1990. Characterization of Na^+-independent Mg^{2+} efflux from erythrocytes. *FEBS Lett.* **271:**149–151
25. Günther, T., Vormann, J., Averdunk, R. 1986. Characterization of furosemide-sensitive Mg^{2+} influx in Yoshida ascites tumor cells. *FEBS Lett.* **197:**297–230
26. Günther, T., Vormann, J., Höllriegl, V. 1990. Characterization of Na^+-dependent Mg^{2+} efflux from Mg^{2+}-loaded rat erythrocytes. *Biochim. Biophys. Acta* **1023:**455–461
27. Halm, D.R., Krasny, E.J., Jr., Frizzel, R.A. 1985. Electrophysiology of flounder intestinal mucosa I. Conductance properties of the cellular and paracellular pathways. *J. Gen. Physiol.* **85:**843
28. Hickman, C.P., Jr. 1968. Ingestion, intestinal absorption, and elimination of seawater and salts in the southern flounder, *Paralichthys lethostigma. Can. J. Zool.* **46:**457–466
29. Hopfer, U., Nelson, K., Perrotto., J., Isselbacher, K.J. 1973. Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **248:**25–32
30. Kayne, L.H., Lee, D.B.N. 1993. Intestinal magnesium absorption. *Miner. Electrolyte Metab.* **19:**210–217
31. Knox, D., Cowey, C.B., Adron, J.W. 1981. Studies on the nutrition of salmonid fish. The magnesium requirement of rainbow trout (*Salmo gairdneri*). *Br. J. Nutr.* **45:**137–148
32. Langridge-Smith, J.E., Field, M. 1981. Sulfate transport in rabbit ileum: characterization of the serosal border anion exchange process. *J. Membrane Biol.* **63:**207–214
33. Mircheff, A.K., Wright, E.M. 1976. Analytical isolation of plasma membranes of intestinal epithelial cells: identification of Na,K-ATPase rich membranes and the distribution of enzyme activities. *J. Membrane Biol.* **28:**309–333
34. Ogino, C., Takashima, F., Chiou, J.Y. 1978. Requirement of rainbow trout for dietary magnesium. *Bull. Jap. Soc. Scient. Fish.* **44:**1105–1108
35. Quamme, G.A., Dirks, J.H. 1980. Magnesium transport in the nephron. *Am. J. Physiol.* **239:**F393–F401
36. Ramos, M.M.P., Ellory, J.C. 1981. Na and Cl transport across the

isolated anterior intestine of the plaice *Pleuronectes platessa*. *J. Exp. Biol.* **90:**123–142

37. Reigh, R.C., Robinson, E.H., Brown, P.B. 1991. Effects of dietary magnesium on growth and tissue magnesium content of blue tilapia *Oreochromis aureus*. *Aquaculture* **22:**192–200
38. Renfro, J.L., Shustock, E. 1985. Peritubular uptake and brush border transport of ^{28}Mg by flounder renal tubules. *Am. J. Physiol.* **249:**F497–F506
39. Schoenmakers, T.J.M., Visser, G.J., Flik, G., Theuvenet, A.P.R. 1992. CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. *BioTechniques* **12:**870–879
40. Shim, K.F., Ng, S.H. 1988. Magnesium requirement of the guppy (*Poecilia reticulata* Peters). *Aquaculture* **73:**131–141
41. Sillén, L.G., Martell, A.E. 1964. Stability Constants of Ion-Metal Complexes. Special publication no. 17. The Chemical Society, London
42. Van der Velden, J.A., Flik, G., Spanings, F.A.T., Verburg, T.G., Kolar, Z.I., Wendelaar Bonga, S.E. 1992. Physiological effects of low-magnesium feeding in the common carp, Cyprinus carpio. *J. Exp. Zool.* **264:**237–244
43. Van der Velden, J.A., Groot, J.A., Flik, G., Polak, P., Kolar, Z.I. 1990. Magnesium transport in fish intestine. *J. Exp. Biol.* **152:**587–592
44. Van der Velden, J.A., Kolar, Z., Flik, G., De Goeij, J.J.M., Wendelaar Bonga, S.E. 1989. Magnesium distribution in freshwater tilapia. *Magnesium Bull.* **11:**28–33
45. Van der Velden, J.A., Spanings, F.A.T., Flik, G., Zegers, C., Kolar, Z.I., Wendelaar Bonga, S.E. 1991. Growth rate and tissue magnesium concentration in adult freshwater tilapia, *Oreochromis mossambicus* (Peters), fed diets differing in magnesium content. *J. Fish Biol.* **39:**83–91
46. White, J.F. 1980. Bicarbonate-dependent chloride absorption in small intestine: ion fluxes and intracellular chloride activities. *J. Membrane Biol.* **53:**95–107
47. Xu, W., Willis, J.S. 1994. Sodium transport through the amiloride-sensitive Na-Mg pathway of hamster red cells. *J. Membrane Biol.* **141:**277–287
48. Zuidema, T., Van Riel, J.W., Siegenbeek van Heukelom, J. 1985. Cellular and transepithelial responses of goldfish intestinal epithelium to chloride substitutions. *J. Membrane Biol.* **88:**293–304