

Mg²⁺ TRANSPORT IN PLASMA MEMBRANE VESICLES OF RENAL EPITHELIUM OF THE MOZAMBIQUE TILAPIA (*OREOCHROMIS MOSSAMBICUS*)

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Summary

To elucidate the mechanisms involved in Mg²⁺ transport at the apical and basolateral poles of the renal tubular epithelium, apical and basolateral plasma membrane vesicle preparations were derived from kidney tissue of freshwater- and seawater-adapted Mozambique tilapia *Oreochromis mossambicus*. Brush-border preparations were enriched 15.8-fold in alkaline phosphatase activity and consisted almost exclusively of right-side-out membrane vesicles. Basolateral membrane preparations were enriched 7.5-fold in Na⁺/K⁺-ATPase activity and contained resealed vesicles and leaky membrane fragments. Mg²⁺ association with brush-border and basolateral plasma membranes, traced using radioactive ²⁷Mg, occurred in an osmotically active space. In all instances, Mg²⁺ binding to the vesicular membrane was low compared with the vesicular uptake. Mg²⁺ equilibration across the vesicular membrane of brush-border preparations was rapid and sensitive to the presence of extravascular Ca²⁺, suggesting that the apical membrane

of the renal epithelium contains a transport pathway for divalent cations. Application of various ionic gradients did not affect vesicular Mg²⁺ transport in apical and basolateral membrane preparations, suggesting the presence of an ion-coupled transport mechanism. ATP or ATP-γ-S did not stimulate Mg²⁺ fluxes, indicating that Mg²⁺ transport does not proceed via an ATP-driven or activated transporter. In these aspects, vesicular Mg²⁺ transport was similar in seawater and freshwater preparations. These results suggest that the apical membrane of renal epithelial cells lacks an active secretory Mg²⁺ transport mechanism. We propose that the Mg²⁺ conductivity of the apical membrane reflects a route for downhill Mg²⁺ entry and is involved in renal Mg²⁺ reabsorption.

Key words: *Oreochromis mossambicus*, Mg²⁺ transport, kidney, plasma membrane, brush border, ATP

Introduction

The kidneys of fish are versatile organs, their contribution to the hydromineral balance depending on the salinity of the ambient medium. In fresh water, their main function is the excretion of excess water and the restriction of mineral losses. Salts are therefore reabsorbed by the tubular epithelium of the nephron, resulting in the production of a voluminous, plasma-hypotonic urine. Conversely, in saline environments, water is taken in by drinking and the volume of water excreted renally is substantially reduced to compensate for osmotic water loss. Excess salts taken in by diffusion across the body wall and by ingestion of sea water are excreted *via* the gills and *via* the kidneys. Whereas the gills are primarily responsible for the excretion of monovalent ions, the kidneys perform a significant role in the excretion of Mg²⁺ and SO₄²⁻ (Hickman, 1968).

In mammals, the proximal tubule and the ascending limb of the loop of Henle are the principal sections of the nephron involved in Mg²⁺ transport (Ryan, 1990; Quamme, 1993). Mg²⁺ reabsorption proceeds predominantly through solute-

linked, paracellular transport and is driven by the transepithelial potential (Di Stefano *et al.* 1993; De Rouffignac and Quamme, 1994). A minor transcellular component may also be involved (Ryan, 1990). In freshwater fish proximal tubules, transepithelial potentials similar to those in mammals have been reported (Nishimura and Imai, 1982) and may constitute a driving force for Mg²⁺ reabsorption. However, reabsorption of water must be limited in freshwater fish, and this will reduce the extent to which paracellular solute-linked transport of salts occurs. Therefore, Mg²⁺ reabsorption in fish probably involves active, transcellular transport.

For the most part, the renal excretion of Mg²⁺ from seawater fish proceeds through tubular secretion (Hickman and Trump, 1969). The glomerular filtration rate is usually low (or glomerular filtration is even absent in aglomerular species) and accounts for only a minor portion of the total Mg²⁺ output. Tubular Mg²⁺ secretion results in urine Mg²⁺ levels well above the levels in the body fluids. Net Mg²⁺ secretion has also been

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demonstrated in isolated proximal tubules (Beyenbach, 1982; Cliff *et al.* 1986). This, together with the low potential difference across the tubular epithelium *in vivo*, suggests that electrically driven transport cannot account for the apparent Mg^{2+} accumulation in urine. It is plausible, therefore, that Mg^{2+} secretion proceeds through a transcellular, active pathway. As epithelial cells maintain a potential difference of approximately -60 mV across the luminal membrane, it is evident that the extrusion of Mg^{2+} across this barrier would require the input of metabolic energy. Intriguingly, the secretory mechanism also seems to be present in the kidneys of freshwater-adapted euryhaline species (Cliff and Beyenbach, 1992). Accordingly, freshwater-acclimated euryhaline species retain the capacity for rapid Mg^{2+} excretion. For instance, injection of freshwater trout with a large dose of Mg^{2+} leads to a sharp and instantaneous increase in urinary Mg^{2+} levels (Oikari and Rankin, 1985). This indicates that the secretory mechanisms are dormant in freshwater fish, but can be activated rapidly, probably triggered directly by the increase in plasma Mg^{2+} levels.

In seawater fish, the urinary Mg^{2+} and Na^+ concentrations are inversely related, and it was suggested that Mg^{2+} secretion may be linked to Na^+ transport *via* an exchange mechanism (Natochin and Gusev, 1970). The Na^+ gradient across the apical membrane carries sufficient energy for uphill Mg^{2+} transport. Furthermore, Na^+ -coupled Mg^{2+} transport has already been demonstrated in several other non-epithelial cell types, most notably erythrocytes (Günther and Vormann, 1985; Lüdi and Schatzmann, 1987; Flatman and Smith, 1990). Therefore, in the few attempts that have been made to elucidate the mechanism of renal Mg^{2+} secretion, attention has mainly focused on the role of Na^+ (Renfro and Shustock, 1985; Cliff *et al.* 1986; Beyenbach *et al.* 1993). Although an inverse relationship between the Mg^{2+} and Na^+ content of the lumen has been confirmed in isolated proximal tubules (Cliff *et al.* 1986), the presence of a Na^+ -dependent Mg^{2+} transport mechanism in the luminal membrane of the tubules has never been demonstrated unequivocally.

The involvement of other mechanisms has hardly been investigated. Hentschel and Zierold (1994) demonstrated Mg^{2+} -containing secretory vesicles located at the apical pole of epithelial cells of dogfish (*Scyliorhinus caniculus*) proximal tubules. Mg^{2+} secretion through exocytosis is compatible with the inhibition of Mg^{2+} transport in isolated renal tubules of the flounder (*Pleuronectes americanus*) by cytochalasin B, which may inhibit vesicular migration (Renfro and Shustock, 1985). Other possible mechanisms would involve the coupling of Mg^{2+} translocation to downhill movement of ions other than Na^+ or to ATP hydrolysis. Anion-dependent Mg^{2+} transport, for instance, has been described for Yoshida ascites tumour cells (Günther *et al.* 1986), hepatocytes (Günther and Höllriegel, 1993), erythrocytes (Günther and Vormann, 1990) and tilapia enterocytes (Bijvelde *et al.* 1996).

Studies on isolated renal tubules have been hampered by the inability to discriminate cellular from paracellular transport (Cliff and Beyenbach, 1992). To circumvent this problem, in

the present study, aspects of transcellular Mg^{2+} transport are studied at a subcellular level. We have isolated the basolateral and apical plasma membrane fractions of kidney epithelial tissue from the euryhaline teleost fish *Oreochromis mossambicus* (Mozambique tilapia). Mg^{2+} transport in isolated membrane vesicles was followed, by means of a radiotracer technique, using ^{27}Mg . The presence of ATP- or ion-gradient-driven Mg^{2+} transport was investigated.

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 ($[Mg^{2+}]=0.3\text{ mmol l}^{-1}$). Fish were adapted to artificial sea water (final density 1022 g l^{-1} ; Wimex sea salt, Wiegandt GmbH and Co., Krefeld, Germany) over a 3 day period by gradual infusion of sea water into tanks containing fresh water. Fish were kept for at least 3 weeks in full-strength sea water before use. The water temperature was $25\text{--}28\text{ }^{\circ}\text{C}$, and the photoperiod was 12 h:12 h light:dark. Fish were fed Trouvit fish pellets (Trouw & Co., Putten, The Netherlands) at a daily rate of 1.5% of the fish total mass.

Membrane isolation

Brush-border membranes were isolated according to a modification of the method described by Booth and Kenny (1974). A fish, weighing approximately 200 g, was killed by spinal transection. The abdominal cavity was cut open lengthways, and the intestinal tract and swim-bladder removed. The kidneys were perfused *in situ* with an ice-cold isotonic solution containing 250 mmol l^{-1} sucrose, 10 mmol l^{-1} Hepes, 1 mmol l^{-1} 1,4-dithiothreitol (DTT), 100 i.u. ml^{-1} aprotinin and 100 i.u. ml^{-1} heparin, adjusted to pH 7.8 with Tris. All further steps were performed at $0\text{--}4\text{ }^{\circ}\text{C}$. Kidney tissue was gently excised and cut into slices approximately 3 mm thick. The renal tissue of two fish was pooled and disrupted using a Dounce homogeniser equipped with a loosely fitting pestle (30 strokes) in 20 ml of a hypotonic mannitol solution, containing 10 mmol l^{-1} mannitol, 1 mmol l^{-1} DTT, 100 i.u. ml^{-1} aprotinin, 2 mmol l^{-1} Tris/HCl, pH 7.4. The resulting tissue suspension was filtered over cheesecloth, and $CaCl_2$ was added to a final concentration of 10 mmol l^{-1} . Membrane precipitation was allowed to proceed for 20 min in an ice-bath under gentle stirring. The suspension was centrifuged for 10 min at 1000 g (Jouan CR3000, CD4 rotor, $2000\text{ revs min}^{-1}$) to remove cellular debris. After centrifugation for 10 min at 7000 g (Beckman L55, 70.1 Ti rotor, $10\,000\text{ revs min}^{-1}$), the resulting supernatant was centrifuged for 30 min at $45\,000\text{ g}$ (70.1 Ti rotor, $25\,000\text{ revs min}^{-1}$) to collect the plasma membrane fraction. After washing, the plasma membrane fraction was suspended in 0.3–0.5 ml of assay medium by 20 passages through a 23 gauge needle (see below for details of the composition of the assay medium). Protein concentration was determined using a commercial Coomassie Blue reagent kit (BioRad) using bovine serum albumin as a reference

(Bradford, 1976). The protein content of membrane preparations was approximately 0.6 mg ml⁻¹. Preparations were stored for up to 3 days in liquid nitrogen.

Basolateral plasma membranes were isolated as described previously (Bijvelds *et al.* 1995). Briefly, a renal tissue homogenate in a 250 mmol l⁻¹ sucrose solution was centrifuged for 10 min at 1400 g to remove nuclei and cellular debris. The resulting supernatant was brought to 1.36 mol l⁻¹ sucrose by mixing with a concentrated sucrose solution, transferred to centrifuge tubes, and overlaid with a 250 mmol l⁻¹ sucrose solution. After centrifugation for 2 h at 154 000 g, the membranes on the interface of the sucrose solutions were collected and mixed with a solution containing the basic ingredients of the assay medium. The membranes were centrifuged for 30 min at 186,000 g, and the resulting membrane pellet was resuspended by 20 passages through a 23 gauge needle in 0.5 ml of assay medium. Membrane preparations contained approximately 2.1 mg ml⁻¹ protein.

Enzyme assays

The marker enzymes used were alkaline phosphatase and aminopeptidase for brush-border membranes (Pfleiderer, 1970; George and Kenny, 1973), NADH-dependent cytochrome *c* reductase for endoplasmic reticulum (Omura and Takesue, 1970), Na⁺/K⁺-ATPase for basolateral plasma membranes (Mircheff and Wright, 1976), thiamine pyrophosphatase for Golgi apparatus fragments (Novikoff and Heus, 1963) and succinic acid dehydrogenase for mitochondrial fragments (Pennington, 1961). Enzyme activities were assayed after membranes had been permeabilised with saponin (0.2 mg mg⁻¹ protein, 10 min at 25 °C) to maximise substrate accessibility.

The percentages of inside-out vesicles and right-side-out vesicles were determined on the basis of acetylcholine esterase activity and glyceraldehyde-3-phosphate-dependent dehydrogenase activity, respectively (Steck, 1974). Triton X-100, at an optimal concentration between 0.010 and 0.020 % (v/v), was used to expose enzyme activity that was masked due to resealing.

Production and measurement of ²⁷Mg

MgO, in which Mg was isotopically enriched in ²⁶Mg to over 97 % (Medgenics Group, Ratingen, Germany and Oak Ridge National Laboratory, Oak Ridge, TN, USA), was dissolved in diluted 'Suprapur' acetic acid (Merck, Darmstadt, Germany), resulting in a 50 mmol l⁻¹ solution of Mg(CH₃COO)₂. ²⁷Mg (half-life 9.46 min) was produced by irradiation of this solution in a thermal neutron flux of 4 × 10¹⁶ m⁻² s⁻¹ for 20 min in the Interfaculty Reactor Institute nuclear reactor. The specific activity of the ²⁷Mg preparation directly after irradiation was approximately 70 GBq mol⁻¹. Radioactivity was determined in a Tri-Carb 2750TR/LL liquid scintillation analyser (Packard Instrument Co., Meriden, CT, USA) with an energy window setting from 2 to 1755 keV. The number of counts recorded per sample ranged from 300 (lower threshold) to approximately 1500. Counting rates were corrected for background and radioactive decay. The

radiochemical purity of the ²⁷Mg preparation was assessed on the basis of the γ-ray spectrum of the irradiated solution, determined using a Ge(Li) detector and associated electronics. The absence of radioactive impurities was routinely confirmed by determination of the apparent half-life of the ²⁷Mg preparation, using liquid scintillation counting. From the decay of the ²⁷Mg preparation between 15 and 55 min after irradiation, a half-life of 9.48 ± 0.02 min was derived, a value consistent with the value of 9.46 min reported previously (Lederer and Shirley, 1978).

Mg²⁺ and Ca²⁺ transport in plasma membrane vesicle preparations

Transport of Mg²⁺ and Ca²⁺ was assayed by means of a rapid filtration technique (Hopfer *et al.* 1973).

Equilibrium Mg²⁺ uptake was assayed by diluting (15×) membrane vesicle preparations loaded with 100 mmol l⁻¹ NaNO₃, 100 mmol l⁻¹ mannitol, 4 mmol l⁻¹ NaCH₃COO and 20 mmol l⁻¹ Hepes/Tris (pH 7.4) in assay medium containing 100 mmol l⁻¹ NaNO₃, 20 mmol l⁻¹ Hepes/Tris (pH 7.4), 2.0 mmol l⁻¹ [²⁷Mg](CH₃COO)₂ and 50–200 mmol l⁻¹ mannitol. To assay electrodiffusive Mg²⁺ transport, membrane vesicle preparations loaded with 100 mmol l⁻¹ mannitol, 4 mmol l⁻¹ NaCH₃COO, 20 mmol l⁻¹ Hepes/Tris (pH 7.4) and either 100 mmol l⁻¹ NaNO₃ or 100 mmol l⁻¹ KNO₃ were pre-incubated with 10 μmol l⁻¹ valinomycin for 10 min on ice. Subsequently, the preparations were diluted (15×) in assay medium made up with either a high or a low KNO₃ concentration to create K⁺ diffusion potentials across the vesicular membrane. Mg²⁺ transport was assayed in the presence of 2.0 mmol l⁻¹ [²⁷Mg](CH₃COO)₂.

The ATP-dependency of Mg²⁺ efflux from brush-border membrane vesicles (BBMVs) was assayed by diluting (8×) membrane vesicle preparations loaded with 100 mmol l⁻¹ KNO₃, 100 mmol l⁻¹ mannitol, 20 mmol l⁻¹ Hepes/Tris (pH 7.4), 1 mmol l⁻¹ ATP and 3.6 mmol l⁻¹ [²⁷Mg](CH₃COO)₂ in assay medium containing (final concentration) 100 mmol l⁻¹ KNO₃, 100 mmol l⁻¹ mannitol, 0.5 mmol l⁻¹ [²⁷Mg](CH₃COO)₂ and 20 mmol l⁻¹ Hepes/Tris, pH 7.4. The ATP-dependency of Mg²⁺ uptake in basolateral membrane vesicles (BLMVs) was assayed by diluting (15×) membrane vesicle preparations loaded with 100 mmol l⁻¹ KNO₃, 100 mmol l⁻¹ mannitol and 20 mmol l⁻¹ Hepes/Tris (pH 7.4) in assay medium containing 100 mmol l⁻¹ KNO₃, 100 mmol l⁻¹ mannitol, 1 mmol l⁻¹ ATP, 2.0 mmol l⁻¹ [²⁷Mg](CH₃COO)₂ and 20 mmol l⁻¹ Hepes/Tris, pH 7.4. When ATP-dependent Ca²⁺ transport was assayed, 0.1 mmol l⁻¹ EGTA was added to buffer free Ca²⁺, and [²⁷Mg](CH₃COO)₂ was replaced by MgCl₂. Plasma membrane Ca²⁺ pump activity was assayed by adding 40 kBq ml⁻¹ [⁴⁵Ca]Cl₂ (NEN-Du Pont, 's-Hertogenbosch, The Netherlands) to assay medium with a calculated free Ca²⁺ concentration of 0.5 μmol l⁻¹ (adjusted using CaCl₂). Thapsigargin (1 μmol l⁻¹) was added to inhibit Ca²⁺ transport in membrane vesicles of endoplasmic reticular origin (Bijvelds *et al.* 1995). ATP-dependent transport was defined as the difference between Mg²⁺ and Ca²⁺ fluxes in the

presence and absence of ATP. In the absence of ATP, 0.97 mmol⁻¹ *trans*-1,2-diaminocyclohexane-*N,N,N'*-tetraacetic acid (CDTA) was added to replace ATP as a Mg²⁺ chelator, thus keeping total and free Mg²⁺ concentrations equal under both conditions. In some instances, ATP was replaced by adenosine-5'-*O*-(3-thiotriphosphate) (ATP- γ -S).

The Na⁺-dependency of Mg²⁺ efflux from BBMV's was assayed by diluting (8 \times) membrane vesicle preparations loaded with 100 mmol⁻¹ NaNO₃, 100 mmol⁻¹ mannitol, 1 mmol⁻¹ ATP, 20 mmol⁻¹ Hepes/Tris (pH 7.4) and 3.6 mmol⁻¹ [²⁷Mg](CH₃COO)₂ in assay medium containing 87.5 mmol⁻¹ KNO₃, 12.5 mmol⁻¹ NaNO₃, 100 mmol⁻¹ mannitol, 0.5 mmol⁻¹ [²⁷Mg](CH₃COO)₂ and 20 mmol⁻¹ Hepes/Tris, pH 7.4. The Na⁺-dependency of Mg²⁺ uptake in BLMV's was assayed by diluting (15 \times) membrane vesicle preparations loaded with 100 mmol⁻¹ NaNO₃, 100 mmol⁻¹ mannitol, 20 mmol⁻¹ Hepes/Tris (pH 7.4) in assay medium containing 93.3 mmol⁻¹ KNO₃, 6.7 mmol⁻¹ NaNO₃, 100 mmol⁻¹ mannitol, 1 mmol⁻¹ ATP, 2.0 mmol⁻¹ [²⁷Mg](CH₃COO)₂ and 20 mmol⁻¹ Hepes/Tris, pH 7.4. Na⁺-dependent transport was defined as the difference between Mg²⁺ fluxes in the presence and absence of a transmembrane Na⁺ gradient, using NaNO₃ to replace KNO₃ iso-osmotically. In some instances, Triton X-100 was added (0.010 %, v/v) to disrupt the vesicular space.

The Cl⁻-dependency of Mg²⁺ efflux from BBMV's was assayed by diluting (8 \times) membrane vesicle preparations loaded with 100 mmol⁻¹ NaCl, 100 mmol⁻¹ mannitol, 20 mmol⁻¹ Hepes/Tris (pH 7.4) and 3.6 mmol⁻¹ [²⁷Mg](CH₃COO)₂ in assay medium containing 87.5 mmol⁻¹ NaNO₃, 12.5 mmol⁻¹ NaCl, 100 mmol⁻¹ mannitol, 0.5 mmol⁻¹ [²⁷Mg](CH₃COO)₂ and 20 mmol⁻¹ Hepes/Tris, pH 7.4. The Cl⁻-dependency of Mg²⁺ uptake in BLMV's was assayed by diluting (15 \times) membrane vesicle preparations loaded with 100 mmol⁻¹ NaNO₃, 100 mmol⁻¹ mannitol and 20 mmol⁻¹ Hepes/Tris (pH 7.4) in assay medium containing 93.3 mmol⁻¹ NaCl, 6.7 mmol⁻¹ NaNO₃, 100 mmol⁻¹ mannitol, 2.0 mmol⁻¹

[²⁷Mg](CH₃COO)₂ and 20 mmol⁻¹ Hepes/Tris, pH 7.4. Cl⁻-dependent transport was defined as the difference between Mg²⁺ fluxes in the presence and absence of a transmembrane Cl⁻ gradient, using NaNO₃ to replace NaCl iso-osmotically.

Incubations were carried out at 28 °C in media of 0.11 mol⁻¹ ionic strength. The free Mg²⁺ and Ca²⁺ concentrations were calculated according to Schoenmakers *et al.* (1992), taking into account metal ion chelation with ATP (Sillén and Martell, 1964). The reaction was quenched by addition of 1 ml of ice-cold stop buffer (150 mmol⁻¹ NaNO₃, 1.5 mmol⁻¹ MgCl₂, 0.1 mmol⁻¹ LaCl₃ and 20 mmol⁻¹ Hepes/Tris at pH 7.4) to an incubation volume of 150 μ l. A volume of 1 ml was filtered on 0.45 μ m 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 of scintillation cocktail (Ultima Gold XR; Packard Instrument, Groningen, The Netherlands). ²⁷Mg and ⁴⁵Ca specific radioactivities were determined by counting the radioactivity in 0.100 ml of the remaining 'quenched reaction' suspension.

Calculations and statistics

Values are expressed as mean \pm standard error (S.E.M.). Statistical significances of differences between means were assessed using the two-tailed Student's *t*-test and accepted at *P*<0.05.

Results

Membrane characterisation

In Table 1, the characteristics of the kidney plasma membrane preparation are summarised. The brush-border membrane preparation was enriched 15.8-fold in the apical membrane marker alkaline phosphatase. The alkaline phosphatase specific activity, expressed as the amount of nitrophenol formed at 37 °C and pH 10.4, was 130 \pm 14 μ mol h⁻¹ mg⁻¹ protein (*N*=5). The aminopeptidase

Table 1. Relative recoveries and enrichment of marker enzymes in Mozambique tilapia kidney brush-border (BBM) and basolateral (BLM) plasma membranes

	Recovery (%)		Enrichment	
	BBM	BLM	BBM	BLM
Protein	0.9 \pm 0.2 (5)	1.9 \pm 0.2 (13)	—	—
Alkaline phosphatase	13.2 \pm 0.9 (5)	5.3 \pm 1.7 (4)	15.8 \pm 2.0 (5)	4.0 \pm 0.6 (4)
Aminopeptidase	7.3 \pm 1.1 (3)	3.7 \pm 0.4 (6)	7.3 \pm 1.2 (3)	2.3 \pm 0.2 (6)
NADH-cytochrome <i>c</i> reductase	1.0 \pm 0.3 (5)	5.4 \pm 0.9 (5)	1.0 \pm 0.3 (5)	2.8 \pm 0.5 (5)
Na ⁺ /K ⁺ -ATPase	2.4 \pm 0.3 (5)	15.5 \pm 1.3 (5)	2.6 \pm 0.4 (5)	7.5 \pm 0.4 (5)
Succinic acid dehydrogenase	0.7 \pm 0.4 (5)	2.5 \pm 0.4 (8)	0.4 \pm 0.2 (5)	1.3 \pm 0.3 (8)
Thiamine pyrophosphatase	3.7 \pm 0.8 (5)	ND	3.0 \pm 0.4 (5)	ND

Recovery was calculated as the percentage of the total activity in the plasma membrane fraction relative to the total activity in the initial tissue homogenate.

Enrichment was calculated by dividing the specific activity in the plasma membrane fraction by the specific activity in the initial tissue homogenate.

Values are means \pm S.E.M. (*N*); ND, not determined.

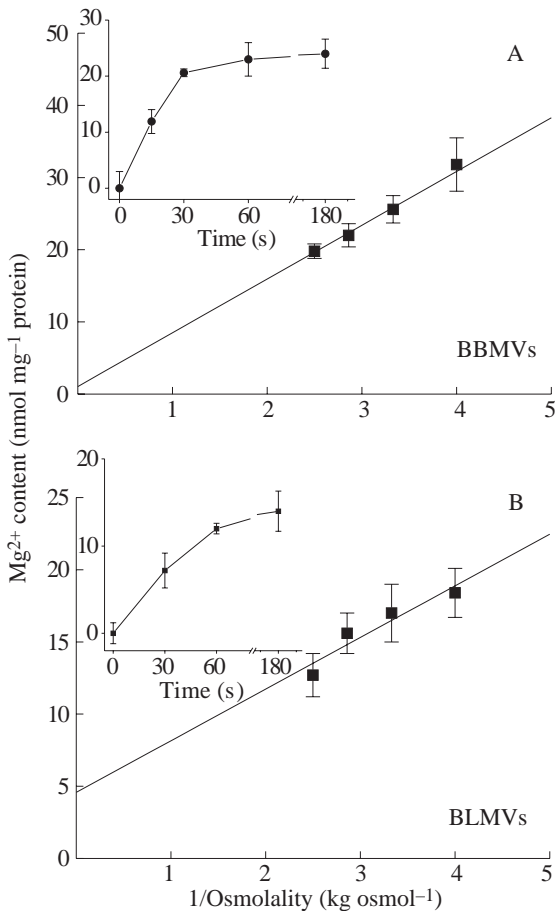


Fig. 1. Equilibrium Mg²⁺ uptake in renal plasma membrane vesicles of the Mozambique tilapia *Oreochromis mossambicus*. Preparations were incubated for 4 min in ionic media (see Materials and methods) at the osmolality indicated, in the presence of 2.0 mmol l⁻¹ Mg²⁺. Regression analysis yielded the following relationship between assay medium osmolality (*Osm*) and Mg²⁺ content (*Q_{Mg}*) of the vesicles: (A) BBMVs, brush-border membrane vesicles: $Q_{Mg} = 1.05 + 7.45/Osm$ ($P = 0.005$; $N = 4$); (B) BLMVs, basolateral membrane vesicles: $Q_{Mg} = 4.58 + 3.58/Osm$ ($P = 0.057$; $N = 4$). The inset shows the time-dependence of Mg²⁺ uptake. Means \pm S.E.M. of three preparations are given.

activity of the brush-border membrane preparation was 7.3-fold higher than that of the initial tissue homogenate. No enrichment was observed in endoplasmic reticular and mitochondrial membrane fragments. The enzyme markers for basolateral plasma membranes (Na⁺/K⁺-ATPase) and Golgi apparatus (thiamine pyrophosphatase) were slightly and significantly enriched.

The percentage of right-side-out BBMVs was 93.2 \pm 1.2 ($N = 5$). The membrane preparation contained virtually no inside-out vesicles (0.3 \pm 0.2%; $N = 5$). From this, we calculate that approximately 6.5% of the membranes are leaky.

Enrichment and recovery of marker enzymes of the basolateral plasma membrane preparation are as reported previously (Bijvelds *et al.* 1995). The orientation of the basolateral plasma membrane preparation was 29.1 \pm 1.8%

Table 2. Effect of ATP, ATP- γ -S and ion gradients on renal plasma membrane Mg²⁺ transport

	Mg ²⁺ release from BBMVs (% of control)	Mg ²⁺ uptake by BLMVs (% of control)
ATP	87 \pm 9	104 \pm 2
ATP- γ -S	94 \pm 6	95 \pm 8
Na ⁺ gradient	106 \pm 5	93 \pm 5
Cl ⁻ gradient	99 \pm 8	98 \pm 5

The Mg²⁺ release from BBMVs and the Mg²⁺ uptake by BLMVs at 60 s are presented as percentages of the paired control value. Values are means \pm S.E.M. ($N = 8$).

inside-out ($N = 5$), 43.9 \pm 2.6% right-side-out ($N = 7$) and, consequently, 27% leaky membranes. In BLMVs from freshwater Mozambique tilapia, a thapsigargin insensitive ATP-dependent Ca²⁺ uptake of 3.9 \pm 0.7 nmol min⁻¹ mg⁻¹ protein ($N = 4$) was demonstrated, confirming that plasma membrane ion transporters remained functional after membrane isolation.

Equilibrium Mg²⁺ uptake

Basal, unstimulated Mg²⁺ uptake reached a plateau after approximately 3 min (Fig. 1, inset); therefore, Mg²⁺ uptake after 4 min of incubation reflects equilibrium uptake. The dependence of equilibrium Mg²⁺ uptake on the osmolality of the suspension medium (a measure of vesicular volume) (Fig. 1) indicates that Mg²⁺ uptake is into the osmotic space. The volume-independent, membrane-bound Mg²⁺ fraction can be estimated from the y-axis intercept of the linear regression fit. At an osmolality of 0.3 osmol kg⁻¹ H₂O, the Mg²⁺ uptake into the osmotic space amounted to 23.4 \pm 3.9 and 12.4 \pm 2.0 nmol mg⁻¹ protein for BBMVs and BLMVs, respectively. On the basis of a free [Mg²⁺] of 2.0 mmol l⁻¹, and assuming that at equilibrium the intravesicular [Mg²⁺] equals the extravesicular [Mg²⁺], we calculate a Mg²⁺ distribution space of 11.7 \pm 2.0 μ l mg⁻¹ protein for BBMVs and 6.2 \pm 1.0 μ l mg⁻¹ protein for BLMVs. The rate of Mg²⁺ uptake by BBMVs was reduced by Ca²⁺: assayed at 30 s, the uptake in the presence of 5 mmol l⁻¹ Ca²⁺ amounted to 46 \pm 7% ($N = 8$; $P < 0.01$) of the paired control value.

Mg²⁺ uptake in BBMVs was voltage-sensitive. An inwardly directed K⁺ gradient in the presence of valinomycin (generating an inside-positive electrical potential across the vesicular membrane) reduced the Mg²⁺ uptake to 85.5 \pm 2.3% ($N = 6$; $P < 0.01$) of the paired control (no K⁺ gradient, but with valinomycin). An outwardly directed K⁺ gradient in the presence of valinomycin stimulated Mg²⁺ uptake to 120.7 \pm 7.6% ($N = 6$; $P < 0.05$). In BLMVs, no voltage-dependent Mg²⁺ transport could be demonstrated.

ATP-dependent or Na⁺- or Cl⁻-coupled Mg²⁺ transport

The effects of ATP and Na⁺ and Cl⁻ gradients on Mg²⁺ transport by renal plasma membrane vesicles were assayed by

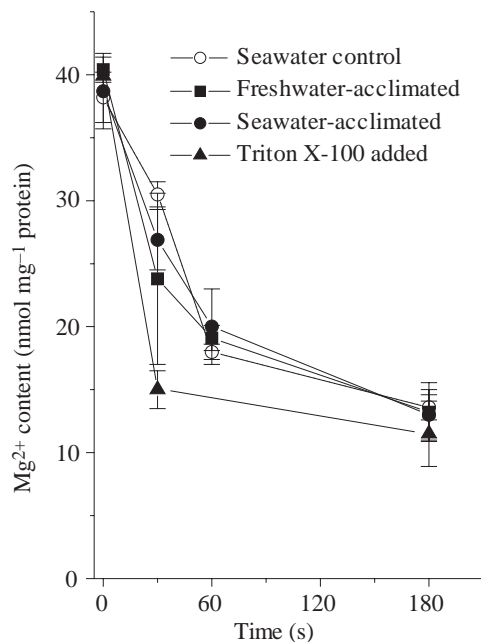


Fig. 2. Na⁺-driven Mg²⁺ transport in renal BBMVs of freshwater- and seawater-adapted Mozambique tilapia *Oreochromis mossambicus*. The graphs show the time course of Mg²⁺ efflux, in the presence (filled symbols) or absence (seawater control, open symbols) of a Na⁺ gradient directed counter to the movement of Mg²⁺. Triton X-100 was added to freshwater membrane preparations to induce maximum efflux. Means \pm S.E.M. of 3–5 preparations are given.

measuring Mg²⁺ release from BBMVs and the Mg²⁺ uptake by BLMVs. In Table 2, the effects of these treatments are summarised. ATP at 1 mmol l⁻¹ did not stimulate Mg²⁺ transport by renal plasma membrane vesicles. Replacement of ATP by its non-hydrolysable analogue ATP- γ -S (1 mmol l⁻¹) did not affect Mg²⁺ transport significantly. Na⁺/Mg²⁺ antiport activity was assayed in the presence of 1 mmol l⁻¹ ATP and a counter-directed Na⁺ gradient to drive Mg²⁺ efflux from BBMVs or Mg²⁺ influx into BLMVs. Mg²⁺ transport was not stimulated by a Na⁺ gradient, and ATP had no effect on this process. Seawater acclimation did not stimulate Mg²⁺ efflux from BBMVs in the presence of an inwardly directed Na⁺ gradient (Fig. 2). Cl⁻-coupled Mg²⁺ transport was assayed in the presence of a Cl⁻ gradient directed parallel to the movement of Mg²⁺ (Bijvelts *et al.* 1996). Neither Mg²⁺ efflux from BBMVs nor Mg²⁺ uptake into BLMVs was stimulated by a Cl⁻ gradient.

Discussion

Although *in vivo* measurements of renal fluid and urine Mg²⁺ levels suggest the presence of an active, energy-consuming Mg²⁺ transporter, we found no evidence for such activity in isolated plasma membrane preparations from kidney tissue. Mg²⁺ fluxes across the basolateral and luminal membranes of the renal epithelium of the Mozambique tilapia appear to be independent of ATP and of Na⁺ and Cl⁻ gradients.

The demonstration of the presence of a Ca²⁺-sensitive Mg²⁺ transport pathway and the absence of an active transport mechanism suggest that Mg²⁺ moves passively across the tubular epithelium, driven by its electrochemical gradient. Whilst Mg²⁺ absorption may indeed be driven by the prevailing electrical potential across the tubular epithelium of freshwater-acclimated fish, the low transepithelial potentials observed in Mg²⁺-excreting seawater fish does not support voltage-driven Mg²⁺ secretion.

Plasma membrane characteristics

The basolateral plasma membrane preparation used in this study was characterised by a 7.5-fold enrichment of the marker enzyme Na⁺/K⁺-ATPase and a plasma membrane Ca²⁺-ATPase activity that may be involved in Ca²⁺ reabsorption (Bijvelts *et al.* 1995). This membrane fraction forms a mixed population of right-side-out and inside-out vesicles and leaky membrane sheets, in accordance with previous studies on basolateral membrane preparations from fish and mammalian kidney (Van Heeswijk *et al.* 1984; Bijvelts *et al.* 1995). The apical plasma membrane preparation, characterised by a 15.8-fold enrichment of the enzyme marker alkaline phosphatase, consists of a population of almost exclusively right-side-out vesicles, in line with a microvillar origin for these membrane fragments (Booth and Kenny, 1974). The enzyme characteristics of this preparation are very similar to those reported for trout (*Oncorhynchus mykiss*) and flounder (*Pleuronectes americanus*) renal brush-border preparations prepared using similar techniques (Renfro and Shustock, 1985; Freire *et al.* 1995).

The membrane vesicles in our preparations were osmotically active and transported Mg²⁺ into the osmotic space. The Mg²⁺ distribution space of BBMVs (11.7 \pm 2.0 μ l mg⁻¹ protein) corresponds well with the equilibrium Mg²⁺ uptake in BBMVs of flounder renal tubules (Renfro and Shustock, 1985), from which we calculate a distribution space of approximately 12 μ l mg⁻¹ protein at an osmolality of 0.3 osmol kg⁻¹ H₂O. Taking into account that a markedly lower Mg²⁺ concentration was used to assay Mg²⁺ transport in the study on flounder, the characteristics of the basal Mg²⁺ influx in renal brush-border preparations from Mozambique tilapia and flounder are comparable. However, a markedly smaller Mg²⁺ distribution space of approximately 3 μ l mg⁻¹ protein was reported for rainbow trout (*Oncorhynchus mykiss*) renal plasma membrane vesicles, resulting in a significantly lower basal Mg²⁺ uptake (Freire *et al.* 1996). Although a similar procedure for isolating renal plasma membranes was used, a markedly lower enrichment of alkaline phosphatase activity was observed for trout renal plasma membranes in the latter study, suggesting that the membrane preparations from trout and Mozambique tilapia differ in their enzymatic make-up and, probably, in some other aspects of membrane composition. Such differences in membrane composition may influence the vesicular volume and, consequently, the Mg²⁺ distribution space.

We have successfully measured ATP-driven Ca²⁺ uptake in

BLMV, demonstrating that ion-transport mechanisms in these plasma membranes remain functional after the plasma membrane isolation procedure. The Ca²⁺ transport activity measured corresponds well with the rate of 4.04 nmol min⁻¹ mg⁻¹ protein that we calculated from the free Ca²⁺ concentration in the assay medium (0.5 μmol l⁻¹) and the kinetic parameters ($V_m=4.50\pm 0.89$ nmol min⁻¹ mg⁻¹ protein, $K_m=57\pm 17$ nmol l⁻¹) we reported previously for Ca²⁺ pump activities in BLMVs from renal tissue of this species (Bijvelds *et al.* 1995). In BBMVs from trout kidney and Mozambique tilapia intestine isolated using similar Ca²⁺ (Mg²⁺) precipitation techniques, functional glucose (Freire *et al.* 1995) and Ca²⁺ (Klaren *et al.* 1997) transport mechanisms, respectively, were demonstrated. Moreover, a functional anion-coupled Mg²⁺ transporter could be demonstrated in a plasma membrane preparation from the intestine of this species (Bijvelds *et al.* 1996). We conclude, therefore, that it is unlikely that ion carriers at work *in vivo* were inactivated during the isolation procedure.

Na⁺-coupled Mg²⁺ transport

We found no evidence for a Na⁺/Mg²⁺ exchange mechanism in the renal basolateral or apical plasma membrane preparations of the Mozambique tilapia. A Na⁺/Mg²⁺ exchange mechanism was postulated on the basis of studies on flounder which showed that tubular Mg²⁺ transport is sensitive to ouabain and to the replacement of luminal Na⁺ (Renfro and Shustock, 1985). Na⁺/Mg²⁺ exchange controls cellular Mg²⁺ levels in a number of cell types (reviewed by Flatman, 1984, 1991), keeping the intracellular Mg²⁺ activity below the electrochemical equilibrium. It is unclear whether, besides its apparent 'housekeeping' function, this transporter may also be involved in transcellular Mg²⁺ movement in Mg²⁺-transporting epithelia. Mg²⁺ efflux across the renal brush border of trout is independent of luminal Na⁺ (Beyenbach *et al.* 1993), and recently it has been shown that Mg²⁺ transport by kidney BBMVs derived from freshwater trout is insensitive to amiloride, a blocker of Na⁺/Mg²⁺ exchange (Freire *et al.* 1996). Thus, evidence for a direct dependence of renal Mg²⁺ transport on Na⁺ is lacking in fish. From our data, we tentatively conclude that a Na⁺/Mg²⁺ exchange mechanism is not involved in renal Mg²⁺ transport, either in freshwater or in seawater fish. Furthermore, we have shown that addition of ATP, considered to be a necessary cofactor for Na⁺/Mg²⁺ exchange in some cell types (DiPolo and Beaugé, 1988; Frenkel *et al.* 1989), does not stimulate Mg²⁺ transport. This also seems to exclude the presence of a primary active ion pump extruding Mg²⁺ at the expense of ATP. To our knowledge no such ATPase, capable of uphill Mg²⁺ transport, has been described in vertebrates.

Anion-coupled Mg²⁺ transport

Anion symport may render Mg²⁺ secretion electroneutral and may thus be a way to overcome the large potential difference across the luminal membrane opposing the efflux of positive charge. Evidently, Mg²⁺ secretion must be

accompanied by secretion of negative charge, but it is not clear whether there is a direct relationship between Mg²⁺ transport and anion secretion. The predominant anions secreted by the tubules are Cl⁻, SO₄²⁻ and, depending on the nutritional intake, inorganic phosphate (Hickman and Trump, 1969). Mg²⁺ secretion *in vitro* is independent of the presence of SO₄²⁻ (Cliff *et al.* 1986), inorganic phosphate and HCO₃⁻ (Renfro and Shustock, 1985) and seems to be most closely correlated with Cl⁻ secretion (Cliff *et al.* 1986). Both the reabsorption (Nishimura and Imai, 1982) and secretion (Sawyer and Beyenbach, 1985; Cliff and Beyenbach, 1988) of Cl⁻ are sensitive to loop diuretics such as furosemide and bumetanide, which is indicative of a (secondary) active Cl⁻ transport mechanism. Mg²⁺ transport by flounder renal tubules has also been shown to be sensitive to furosemide, suggesting that Mg²⁺ secretion may be linked to Cl⁻ secretion (Renfro and Shustock, 1985). Recently, we have demonstrated that Cl⁻-dependent Mg²⁺ transport occurs in the intestinal epithelium of the Mozambique tilapia (Bijvelds *et al.* 1996). This pathway, however, is insensitive to furosemide, and in the present study we could show no effect of Cl⁻ substitution on vesicular Mg²⁺ fluxes. Therefore, there is no strong evidence to support a direct coupling between Cl⁻ and Mg²⁺ secretion in the kidney of the Mozambique tilapia.

Voltage- and Ca²⁺-sensitive Mg²⁺ transport

Assuming that tubular Mg²⁺ secretion must proceed *via* an energised, transcellular mechanism, we predicted that active Mg²⁺ transport would occur across the apical plasma membrane of the renal epithelium. However, the present study suggests that Mg²⁺ transport across the brush-border membrane is insensitive to ATP and to transmembrane gradients of Na⁺ or Cl⁻ and is mediated by a Ca²⁺- and voltage-sensitive Mg²⁺ conductive pathway that allows Mg²⁺ transport down its electrochemical gradient. It has previously been shown that tubular Mg²⁺ transport in the marine flounder is inhibited by Ca²⁺, and that a transmembrane potential difference across the luminal membrane stimulates Mg²⁺ fluxes (Renfro and Shustock, 1985). Similar Ca²⁺-sensitive, voltage-driven Mg²⁺ transport has been reported for the kidney epithelium of freshwater trout (Freire *et al.* 1996). Equilibration of Mg²⁺ across the renal brush-border membrane proceeded relatively quickly. The high Mg²⁺ permeability observed *in vitro* may indicate the presence of a high-capacity Mg²⁺ transport pathway in the luminal membrane of the renal epithelium. Because of the prevailing potential difference across the apical pole of the tubular cells, such a transport system could constitute a mechanism for Mg²⁺ entry, but not for Mg²⁺ extrusion. Therefore, its primary role may be in the regulation of cellular Mg²⁺ concentration and/or in Mg²⁺ reabsorption.

Tubular Mg²⁺ secretion and reabsorption

Our data indicate that the luminal membrane of the renal epithelium lacks an active Mg²⁺ extrusion mechanism capable of uphill Mg²⁺ secretion. In accordance with the functional

similarity between proximal tubules from freshwater and seawater fish (Cliff and Beyenbach, 1992), Mg^{2+} fluxes were similar in freshwater and seawater vesicle preparations and no ATP-driven or ion gradient-activated Mg^{2+} transport was apparent. It has been suggested that Mg^{2+} secretion occurs *via* vesicular transport. The presence of microcrystalline aggregates in cell organelles of flounder proximal tubules and luminal fluid (Hickman and Trump, 1969), and the discovery of apically located Mg^{2+} -loaded vesicles in cells of dogfish proximal tubules (Hentschel and Zierold, 1994), indicates that Mg^{2+} may be accumulated and stored (either precipitated or glycoprotein-bound) in subcellular compartments. Although there is no sound evidence that these vesicles discharge their contents across the brush border into the tubular lumen, the presence of salt precipitates (mainly the highly insoluble phosphate salts of the divalent cations) in renal fluid and urine of Mg^{2+} -secreting fish is compatible with such a mechanism (Pitts, 1934; Hickman, 1968; Hickman and Trump, 1969; Maren *et al.* 1992).

Although Mg^{2+} secretion must be a transcellular, and thus active, process, there seems to be no obvious requirement for an active transcellular Mg^{2+} absorption route. NaCl absorption generates a lumen-positive potential difference (Nishimura and Imai, 1982) that may drive the reabsorption of divalent cations in fish renal tubules, as in the mammalian kidney (Di Stefano *et al.* 1993; Friedman and Geseke, 1993). The transport pathways described for mammalian kidney cells seem to be primarily involved in the control of cellular Mg^{2+} levels (Quamme, 1993) and, in contrast to the mechanisms described for fish kidney (Renfro and Shustock, 1985; Freire *et al.* 1996), are insensitive to high external Ca^{2+} concentrations (Quamme and Dai, 1990). As yet, no active transport mechanism has been demonstrated in mammalian kidney cells that could mediate Mg^{2+} reabsorption. We also found no evidence for an active Mg^{2+} transporter in the basolateral membrane of renal cells that could transport Mg^{2+} uphill from the cellular compartment to the peritubular fluid. Although not necessarily required for Mg^{2+} reabsorption, such a mechanism may be required for maintaining cellular Mg^{2+} homeostasis. As intracellular Mg^{2+} is strongly buffered and changes in intracellular Mg^{2+} levels are therefore moderate and occur only slowly, a mechanism of low capacity would suffice. We cannot exclude that such a transport mechanism may have gone undetected in our assays because of its low activity.

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