Mg^2+ 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 Mg^2+ 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. Mg^2+ association with brush-border and basolateral plasma membranes, traced using radioactive 27Mg, occurred in an osmotically active space. In all instances, Mg^2+ binding to the vesicular membrane was low compared with the vesicular uptake. Mg^2+ equilibration across the vesicular membrane of brush-border preparations was rapid and sensitive to the presence of extravesicular Ca^2+, 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 Mg^2+ transport in apical and basolateral membrane preparations, suggesting the presence of an ion-coupled transport mechanism. ATP or ATP-γ-S did not stimulate Mg^2+ fluxes, indicating that Mg^2+ transport does not proceed via an ATP-driven or activated transporter. In these aspects, vesicular Mg^2+ transport was similar in seawater and freshwater preparations. These results suggest that the apical membrane of renal epithelial cells lacks an active secretory Mg^2+ transport mechanism. We propose that the Mg^2+ conductivity of the apical membrane reflects a route for downhill Mg^2+ entry and is involved in renal Mg^2+ reabsorption.

Key words: Oreochromis mossambicus, Mg^2+ 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 Mg^2+ and SO_4^{2-} (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 Mg^2+ transport (Ryan, 1990; Quamme, 1993). Mg^2+ 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 Mg^2+ 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, Mg^2+ reabsorption in fish probably involves active, transcellular transport.

For the most part, the renal excretion of Mg^2+ 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 Mg^2+ output. Tubular Mg^2+ secretion results in urine Mg^2+ levels well above the levels in the body fluids. Net Mg^2+ secretion has also been

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electrically driven transport cannot account for the apparent
demonstrated in isolated proximal tubules (Beyenbach, 1982; Clifford 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 Mg2+ accumulation in urine. It is plausible, therefore, that Mg2+ 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 Mg2+ 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 Mg2+ excretion. This, together with the low potential difference in the luminal membrane, suggests that Mg2+ is excluded from the cell by a gradient-dependent mechanism.

Therefore, in the few attempts that have been made to elucidate the mechanism of renal Mg2+ 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 Mg2+ and Na+ content of the lumen has been confirmed in isolated proximal tubules (Cliff et al. 1986), the presence of a Na+-dependent Mg2+ 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 Mg2+-containing secretory vesicles located at the apical pole of epithelial cells of dogfish (Scyliorhinus caniculus) proximal tubules. Mg2+ secretion through exocytosis is compatible with the inhibition of Mg2+ 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 Mg2+ translocation to downhill movement of ions other than Na+ or to ATP hydrolysis. Anion-dependent Mg2+ 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 (Bijvelds 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 Mg2+ 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). Mg2+ transport in isolated membrane vesicles was followed, by means of a radiotracer technique, using 22Mg. The presence of ATP- or ion-gradient-driven Mg2+ 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 ([Mg2+] = 0.3 mmol l-1). Fish were adapted to artificial sea water (final density 1.022 g l-1; Wimex 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 25-28 °C, and the photoperiod was 12:12 h light:dark. Fish were fed Trouvif 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 Heps, 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 0-4 °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 CaCl2 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 revs min-1) to remove cellular debris. After centrifugation for 10 min at 7000 g (Beckman L55, 70.1 Ti rotor, 10,000 revs min-1), the resulting supernatant was centrifuged for 30 min at 45000 g (70.1 Ti rotor, 25,000 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
Mg$^{2+}$ transport in tilapia kidney

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 mM$^{-1}$ sucrose solution was centrifuged for 10 min at 14000 g to remove nuclei and cellular debris. The resulting supernatant was brought to 1.36 M$^{-1}$ sucrose by mixing with a concentrated sucrose solution, transferred to centrifuge tubes, and overlaid with a 250 mM$^{-1}$ sucrose solution. After centrifugation for 2 h at 154000 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 186000 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 (Pfeiferer, 1970; George and Kenny, 1973), NADH-dependent cytochrome c reductase for endoplasmic reticulum (Omura and Takesue, 1970), Na$^+$/K$^+$/ATPase for basolateral plasma membranes (Micheff 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 ml$^{-1}$ 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 $^{27}$Mg**

MgO, in which Mg was isotopically enriched in $^{26}$Mg 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 mM$^{-1}$ solution of Mg(CH$_3$COO)$_2$. $^{27}$Mg (half-life 9.46 min) was produced by irradiation of this solution in a thermal neutron reactor at Brookhaven National Laboratory, Upton, NY, USA, in a 50 mM$^{-1}$ solution of Mg(CH$_3$COO)$_2$.

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 $^{27}$Mg 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 $^{27}$Mg preparation, using liquid scintillation counting. From the decay of the $^{27}$Mg 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).

**$^{27}$Mg and Ca$^{2+}$ transport in plasma membrane vesicle preparations**

Transport of Mg$^{2+}$ and Ca$^{2+}$ was assayed by means of a rapid filtration technique (Hopfer et al. 1973).

Equilibrium Mg$^{2+}$ uptake was assayed by diluting (15×) membrane vesicle preparations loaded with 100 mM$^{-1}$ NaN$_3$, 100 mM$^{-1}$ mannitol, 4 mM$^{-1}$ NaCH$_3$COO and 20 mM$^{-1}$ Hepes/Tris (pH 7.4) in assay medium containing 100 mM$^{-1}$ NaN$_3$, 20 mM$^{-1}$ Hepes/Tris (pH 7.4), 2.0 mM$^{-1}$ $^{27}$Mg(CH$_3$COO)$_2$ and 50–200 mM$^{-1}$ mannitol. To assay electrodiffusive Mg$^{2+}$ transport, membrane vesicle preparations were loaded with 100 mM$^{-1}$ mannitol, 4 mM$^{-1}$ NaCH$_3$COO, 20 mM$^{-1}$ Hepes/Tris (pH 7.4) and either 100 mM$^{-1}$ NaNO$_3$ or 100 mM$^{-1}$ KNO$_3$. Vesicle preparations were stored for up to 3 days in liquid nitrogen. The ATP-dependency of Mg$^{2+}$ efflux from brush-border membrane vesicle preparations (BBMVs) was assayed by diluting (8×) membrane vesicle preparations loaded with 100 mM$^{-1}$ KNO$_3$, 100 mM$^{-1}$ mannitol, 20 mM$^{-1}$ Hepes/Tris (pH 7.4), 1 mM$^{-1}$ ATP and 3.6 mM$^{-1}$ $^{27}$Mg(CH$_3$COO)$_2$ in assay medium containing (final concentration) 100 mM$^{-1}$ KNO$_3$, 100 mM$^{-1}$ mannitol, 0.5 mM$^{-1}$ $^{27}$Mg(CH$_3$COO)$_2$ and 20 mM$^{-1}$ Hepes/Tris, pH 7.4. The ATP-dependency of Mg$^{2+}$ uptake in basolateral membrane vesicles (BLMVs) was assayed by diluting (15×) membrane vesicle preparations loaded with 100 mM$^{-1}$ KNO$_3$, 100 mM$^{-1}$ mannitol and 20 mM$^{-1}$ Hepes/Tris (pH 7.4) in assay medium containing 100 mM$^{-1}$ KNO$_3$, 100 mM$^{-1}$ mannitol, 1 mM$^{-1}$ ATP, 2.0 mM$^{-1}$ $^{27}$Mg(CH$_3$COO)$_2$ and 20 mM$^{-1}$ Hepes/Tris, pH 7.4. When ATP-dependent Ca$^{2+}$ transport was assayed, 0.1 mM$^{-1}$ EGTA was added to buffer free Ca$^{2+}$, and $^{27}$Mg(CH$_3$COO)$_2$ was replaced by MgCl$_2$. Plasma membrane Ca$^{2+}$ pump activity was assayed by adding 40 kBq ml$^{-1}$ $^{45}$CaCl$_2$ (NEN-Du Pont, ‘s-Hertogenbosch, The Netherlands) to assay medium with a calculated free Ca$^{2+}$ concentration of 0.5 mM$^{-1}$ (adjusted using CaCl$_2$). Thapsigargin (1 mM$^{-1}$) was added to inhibit Ca$^{2+}$ transport in membrane vesicles of endoplasmic reticular origin (Bijvelds et al. 1995). ATP-dependent transport was defined as the difference between Mg$^{2+}$ and Ca$^{2+}$ fluxes in the
In the absence of ATP, Mg$^{2+}$ uptake in BLMVs was assayed by diluting (15x) membrane vesicle preparations loaded with 100 mmol$^{-1}$ NaCl, 100 mmol$^{-1}$ mannitol, 20 mmol$^{-1}$ Heps/Tris (pH 7.4) and 3.6 mmol$^{-1}$ $[^{27}\text{Mg}](\text{CH}_3\text{COO})_2$ in assay medium containing 87.5 mmol$^{-1}$ KNO$_3$, 12.5 mmol$^{-1}$ NaNO$_3$, 100 mmol$^{-1}$ mannitol, 0.5 mmol$^{-1}$ $[^{27}\text{Mg}](\text{CH}_3\text{COO})_2$ and 20 mmol$^{-1}$ Heps/Tris, pH 7.4. Na$^+$-dependent transport was defined as the difference between Mg$^{2+}$ fluxes in the presence and absence of a transmembrane Na$^+$ gradient, using NaNO$_3$ to replace NaCl iso-osmotically.

Incubations were carried out at 28°C in media of 0.11 mol$^{-1}$ ionic strength. The free Mg$^{2+}$ and Cl$^-$ concentrations were calculated according to Schoenmakers et al. (1992), taking into account metal ion chelation with ATP$\gamma$S (Sillén and Martell, 1964). The reaction was quenched by the addition of 1 ml of ice-cold stop buffer (150 mmol$^{-1}$ NaNO$_3$, 1.5 mmol$^{-1}$ MgCl$_2$, 0.1 mmol$^{-1}$ LaCl$_3$ and 20 mmol Heps/Tris at pH 7.4.) to an incubation volume of 150 µl. The volume of 1 ml was filtered on 0.45 µm ME25 membrane filter (Schleicher & Schuell, Dassel, Germany) at a reduced pressure of 75 kPa. Filters were rinsed twice with 2 ml of stop buffer (150 mmol$^{-1}$ NaNO$_3$, 1.5 mmol$^{-1}$ MgCl$_2$, 0.1 mmol$^{-1}$ LaCl$_3$ and 20 mmol Heps/Tris at pH 7.4.) and transferred to 10 ml of scintillation cocktail (Ultima G, XR: Packard Instrument, Groningen, The Netherlands). $^{27}$Mg and $^{45}$Ca 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 ± standard error (s.E.M.). Statistical significances of differences between means were assessed using the two-tailed Student's t-test and accepted when $p<0.05$.

Results

Membrane characterisation
In Table 1, the characteristics of the kidney plasma membranes 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, 130±14 µmol h$^{-1}$ mg$^{-1}$ protein (N=5). The aminopeptidase

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

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 ± s.e.m. (N); ND, not determined.
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±1.2 (N=5). The membrane preparation contained virtually no inside-out vesicles (0.3±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±1.8% inside-out (N=5). 43.9±2.6% right-side-out (N=7) and, consequently, 27% leaky membranes. In BBMVs from freshwater Mozambique tilapia, a thapsigargin insensitive ATP-dependent Ca2+ uptake of 3.9±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 v-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±3.9 and 12.4±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±2.0 μl mg⁻¹ protein for BBMVs and 6.2±1.0 μl 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±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±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±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
counter-directed Na\(^+\) gradient to drive Mg\(^{2+}\) efflux from appear to be independent of ATP and of Na\(^+\) and Cl\(^-\) gradients. Means ± s.e.m. of 3–5 preparations are given.

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

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

Discussion

Although in vivo measurements of renal fluid and urine Mg\(^{2+}\) levels suggest the presence of an active, energy-consuming Mg\(^{2+}\) transporter, we found no evidence for such activity in isolated plasma membrane preparations from kidney tissue. Mg\(^{2+}\) 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 Ca\(^{2+}\)-sensitive Mg\(^{2+}\) transport pathway and the absence of an active transpo mechanism suggest that Mg\(^{2+}\) moves passively across the tubular epithelium, driven by its electrochemical gradient. Whilst Mg\(^{2+}\) absorption may indeed be driven by the prevailing electrical potential across the tubular epithelium of freshwater acclimated fish, the low transepithelial potentials observed in Mg\(^{2+}\)-excreting seawater fish does not support voltage-driven Mg\(^{2+}\) 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 Ca\(^{2+}\) ATPase activity that may be involved in Ca\(^{2+}\) reabsorption (Bijvelds et al. 1995). This membrane fraction forms a mixed population of right-side-out and inside-out vesicles and leaflet membrane sheets, in accordance with previous studies of basolateral membrane preparations from fish and mammalian kidney (Van Heeswijk et al. 1984; Bijvelds et al. 1995). The apical plasma membrane preparation, characterised by a 15-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 enzym characteristics of this preparation are very similar to those reported for trout (Oncorhynchus mykiss) and flounder (Platichthys flesus) renal brush-border preparation prepared using similar techniques (Renfro and Shustock, 1983; Freire et al. 1995).

The membrane vesicles in our preparations were osmotically active and transported Mg\(^{2+}\) into the osmotic space. The Mg distribution space of BBMVs (11.7±2.0 μg mg\(^{-1}\) protein) corresponds well with the equilibrium Mg\(^{2+}\) uptake in BBMVs of flounder renal tubules (Renfro and Shustock, 1985), from which we calculate a distribution space of approximately 12 μg mg\(^{-1}\) protein at an osmolality of 0.3 osmol kg\(^{-1}\) H\(_2\)O. Taking into account that a markedly lower Mg\(^{2+}\) concentration was used to assay Mg\(^{2+}\) transport in the study on flounder, the characteristics of the basal Mg\(^{2+}\) influx in renal brush-bord preparations from Mozambique tilapia and flounder are comparable. However, a markedly smaller Mg\(^{2+}\) distribution space of approximately 3 μg mg\(^{-1}\) protein was reported for rainbow trout (Oncorhynchus mykiss) renal plasma membrane vesicles, resulting in a significantly lower basal Mg\(^{2+}\) 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 in trout renal plasma membranes in the latter study, suggesting that the membrane preparations from trout and Mozambiq tilapia differ in their enzymatic make-up and, probably, some other aspects of membrane composition. Such differences in membrane composition may influence vesicular volume and, consequently, the Mg\(^{2+}\) distribution space.

We have successfully measured ATP-driven Ca\(^{2+}\) uptake
BLMVs, demonstrating that ion-transport mechanisms in these plasma membranes remain functional after the plasma membrane isolation procedure. The Ca\(^{2+}\) transport activity measured corresponds well with the rate of 4.04 nmol min\(^{-1}\) mg\(^{-1}\) protein that we calculated from the free Ca\(^{2+}\) concentration in the assay medium (0.5 \(\mu\)mol L\(^{-1}\)) and the kinetic parameters (\(V_m=4.50\pm0.89\ \text{nmol min}^{-1}\ \text{mg}^{-1}\ \text{protein}, \ K_m=57\pm17\ \text{nmol L}^{-1}\)) we reported previously for Ca\(^{2+}\) pump activities in BLMVs from renal tissue of this species (Bijvelds et al. 1995). In BBMV preparations from trout kidney and Mozambique tilapia intestine isolated using similar Ca\(^{2+}\) (Mg\(^{2+}\)) precipitation techniques, functional glucose (Freire et al. 1995) and Ca\(^{2+}\) (Klaren et al. 1997) transport mechanisms, respectively, were demonstrated. Moreover, a functional anion-coupled Mg\(^{2+}\) 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 Mg\(^{2+}\) transport**

We found no evidence for a Na\(^{+}\)/Mg\(^{2+}\) exchange mechanism in the renal basolateral or apical plasma membrane preparations of the Mozambique tilapia. A Na\(^{+}\)/Mg\(^{2+}\) exchange mechanism was postulated on the basis of studies on flounder which showed that tubular Mg\(^{2+}\) transport is sensitive to ouabain and to the replacement of luminal Na\(^{+}\) (Renfro and Shustock, 1985). Na\(^{+}\)/Mg\(^{2+}\) exchange controls cellular Mg\(^{2+}\) levels in a number of cell types (reviewed by Flatman, 1984, 1991), keeping the intracellular Mg\(^{2+}\) activity below the electrochemical gradient. It is unclear whether, besides its apparent ‘housekeeping’ function, this transporter may also be involved in transcellular Mg\(^{2+}\) movement in Mg\(^{2+}\)-transporting epithelia. Mg\(^{2+}\) efflux across the renal brush border of trout is independent of luminal Na\(^{+}\) (Beyenbach et al. 1993), and recently it has been shown that Mg\(^{2+}\) transport by kidney BBMVs derived from freshwater trout is insensitive to amiloride, a blocker of Na\(^{+}\)/Mg\(^{2+}\) exchange (Freire et al. 1996). Thus, evidence for a direct dependence of renal Mg\(^{2+}\) transport on Na\(^{+}\) is lacking in fish. From our data, we tentatively conclude that a Na\(^{+}\)/Mg\(^{2+}\) exchange mechanism is not involved in renal Mg\(^{2+}\) transport, either in freshwater or in seawater fish. Furthermore, we have shown that addition of ATP, considered to be a necessary cofactor for Na\(^{+}\)/Mg\(^{2+}\) exchange in some cell types (DiPolo and Beaugé, 1988; Frenkel et al. 1989), does not stimulate Mg\(^{2+}\) transport. This also seems to exclude the presence of a primary active ion pump extruding Mg\(^{2+}\) at the expense of ATP. To our knowledge no such ATPase, capable of uphill Mg\(^{2+}\) transport, has been described in vertebrates.

**Anion-coupled Mg\(^{2+}\) transport**

Anion symport may render Mg\(^{2+}\) 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, Mg\(^{2+}\) secretion must be accompanied by secretion of negative charge, but it is not clear whether there is a direct relationship between Mg\(^{2+}\) transport and anion secretion. The predominant anions secreted by the tubules are Cl\(^{-}\), SO\(_4\)\(^{2-}\) and, depending on the nutritional intake, inorganic phosphate (Hickman and Trump, 1969). Mg\(^{2+}\) secretion in vitro is independent of the presence of SO\(_4\)\(^{2-}\) (Cliff et al. 1986), inorganic phosphate and HCO\(_3\)\(^{-}\) (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. Mg\(^{2+}\) transport by flounder renal tubules has also been shown to be sensitive to furosemide, suggesting that Mg\(^{2+}\) secretion may be linked to Cl\(^{-}\) secretion (Renfro and Shustock, 1985). Recently, we have demonstrated that Cl\(^{-}\)-dependent Mg\(^{2+}\) 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 Mg\(^{2+}\) fluxes. Therefore, there is no strong evidence to support a direct coupling between Cl\(^{-}\) and Mg\(^{2+}\) secretion in the kidney of the Mozambique tilapia.

**Voltage- and Ca\(^{2+}\)-sensitive Mg\(^{2+}\) transport**

Assuming that tubular Mg\(^{2+}\) secretion must proceed via an energised, transcellular mechanism, we predicted that active Mg\(^{2+}\) transport would occur across the apical plasma membrane of the renal epithelium. However, the present study suggests that Mg\(^{2+}\) transport across the brush-border membrane is insensitive to ATP and to transmembrane gradients of Na\(^{+}\) or Cl\(^{-}\) and is mediated by a Ca\(^{2+}\)- and voltage-sensitive Mg\(^{2+}\) conductive pathway that allows Mg\(^{2+}\) transport down its electrochemical gradient. It has previously been shown that tubular Mg\(^{2+}\) transport in the marine flounder is inhibited by Ca\(^{2+}\), and that a transmembrane potential difference across the luminal membrane stimulates Mg\(^{2+}\) fluxes (Renfro and Shustock, 1985). Similar Ca\(^{2+}\)-sensitive, voltage-driven Mg\(^{2+}\) transport has been reported for the kidney epithelium of freshwater trout (Freire et al. 1996). Equilibration of Mg\(^{2+}\) across the renal brush-border membrane proceeded relatively quickly. The high Mg\(^{2+}\) permeability observed in vitro may indicate the presence of a high-capacity Mg\(^{2+}\) 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 Mg\(^{2+}\) entry, but not for Mg\(^{2+}\) extrusion. Therefore, its primary role may be in the regulation of cellular Mg\(^{2+}\) concentration and/or in Mg\(^{2+}\) reabsorption.

**Tubular Mg\(^{2+}\) secretion and reabsorption**

Our data indicate that the luminal membrane of the renal epithelium lacks an active Mg\(^{2+}\) extrusion mechanism capable of uphill Mg\(^{2+}\) secretion. In accordance with the functional
similarity between proximal tubules from freshwater and seawater fish (Cliff and Beyenbach, 1992). Mg2+ fluxes were similar in freshwater and seawater vesicle preparations and no ATP-driven or ion gradient-activated Mg2+ transport was apparent. It has been suggested that Mg2+ secretion occurs via vesicular transport. The presence of microcrystalline aggregates in cell organelles of flounder proximal tubules and similar in freshwater and seawater vesicle preparations and no similarity between proximal tubules from freshwater and of apically located Mg2+-loaded vesicles in cells of dogfish proximal tubules (Hentschel and Zierold, 1994), indicates that Mg2+ 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 Mg2+-secreting fish is compatible with such a mechanism (Pitts, 1934; Hickman, 1968; Hickman and Trump, 1969; Maren et al., 1992).

Although Mg2+ secretion must be a transcellular, and thus active, process, there seems to be no obvious requirement for an active transcellular Mg2+ 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 Gesek, 1993). The transport pathways described for mammalian kidney cells seem to be primarily involved in the control of cellular Mg2+ 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 Ca2+ concentrations (Quamme and Dai, 1990). As yet, no active transport mechanism has been demonstrated in mammalian kidney cells that could mediate Mg2+ reabsorption. We also found no evidence for an active Mg2+ transporter in the basolateral membrane of renal cells that could transport Mg2+ uphill from the cellular compartment to the peritubular fluid. Although not necessarily required for Mg2+ reabsorption, such a mechanism may be required for maintaining cellular Mg2+ homeostasis. As intracellular Mg2+ is strongly buffered and changes in intracellular Mg2+ 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.

References


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