Characterization of two Mg$^{2+}$ transporters in sealed plasma membrane vesicles from rat liver

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CEFARATTI, Christie, Andrea Romani, and Antonio Scarpa. Characterization of two Mg$^{2+}$ transporters in sealed plasma membrane vesicles from rat liver. Am. J. Physiol. 275 (Cell Physiol. 44): C995–C1008, 1998.—The plasma membrane of mammalian cells possesses rapid Mg$^{2+}$ transport mechanisms. The identity of Mg$^{2+}$ transporters is unknown, and so are their properties. In this study, Mg$^{2+}$ transporters were characterized using a biochemically and morphologically standardized preparation of sealed rat liver plasma membranes (LPM) whose intravesicular content could be set and controlled. The system has the advantages that it is not regulated by intracellular signaling machinery and that the intravesicular ion milieu can be designed. The results indicate that 1) LPM retain trapped intravesicular total Mg$^{2+}$ with negligible leak; 2) the addition of Na$^+$ or Ca$^{2+}$ induces a concentration- and temperature-dependent efflux corresponding to 30–50% of the intravesicular Mg$^{2+}$; 3) the rate of flux is very rapid (137.6 and 86.8 nmol total Mg$^{2+}$·µm$^{-2}$·min$^{-1}$ after Na$^+$ and Ca$^{2+}$ addition, respectively); 4) coaddition of maximal concentrations of Na$^+$ and Ca$^{2+}$ induces an additive Mg$^{2+}$ efflux; 5) both Na$^+$ and Ca$^{2+}$-stimulated Mg$^{2+}$ effluxes are inhibited by amiloride, imipramine, or quinidine but not by vanadate or Ca$^{2+}$-channel blockers; 6) extracellular Na$^+$ or Ca$^{2+}$ can stimulate Mg$^{2+}$ efflux in the absence of Mg$^{2+}$ gradients; and 7) Mg$^{2+}$ uptake occurs in LPM loaded with Na$^+$ but not with Ca$^{2+}$, thus indicating that Na$^+$/Mg$^{2+}$ but not Ca$^{2+}$/Mg$^{2+}$ exchange is reversible. These data are consistent with the operation of two distinct Mg$^{2+}$ transport mechanisms and provide new information on rates of Mg$^{2+}$ transport, specificity of the cotransported ions, and reversibility of the transport.

sodium/magnesium antiporter; calcium/magnesium antiporter; hepatocytes

MAGNESIUM ION, a major cation within the cell (8, 10), is required for membrane structure stabilization (8, 10), cell cycle regulation (22, 39), structural modification of phosphometabolites (see Refs. 8 and 10 for review), and the functioning of a large number of enzymes (10, 32). The well-documented absence of significant changes in cytosolic free Mg$^{2+}$ (4, 10, 24) following a variety of metabolic interventions previously led to the assumption that Mg$^{2+}$ content remains relatively constant within the cell and to the conclusion that cellular enzymes cannot be regulated in vivo by changes in cytosolic Mg$^{2+}$. On the basis of the findings that large amounts of “total” Mg$^{2+}$ are accumulated or released by cells within a few minutes in response to hormonal stimulation (28–31), this assumption has recently been challenged. Significant experimental evidence obtained initially in nonmammalian cells (e.g., squid axon) and more recently in several mammalian cell types suggests that Mg$^{2+}$ transport across the plasma membrane occurs through at least two mechanisms. A Na$^+$/Mg$^{2+}$ exchanger has been shown to operate in cardiac myocytes (29, 31, 38) as well as in hepatocytes (12, 28, 30), thymocytes (15), and chicken and human erythrocytes (12). This transport mechanism electroneutrally exchanges 1 Mg$^{2+}$ for 2 Na$^+$ in chicken erythrocytes, and possibly in other cell types as well (13). Recently, it was reported that Mg$^{2+}$ extrusion from a variety of cell types is stimulated by cAMP (15, 28, 31, 41), inhibited by amiloride (13, 38), and not operative in the absence of extracellular Na$^+$ (21, 29, 30). The presence of a Na$^+$-independent Mg$^{2+}$ extrusion pathway has also been proposed in human, chicken, and rat erythrocytes (14). However, the requirement for a specific cation to be accumulated in exchange for Mg$^{2+}$ has not been defined. In different experimental models or conditions, cellular Mg$^{2+}$ has been shown to exchange for extracellular Mn$^{2+}$ (6), Ca$^{2+}$ (29), HCO$_3^-$ (12), or other cations or anions (12), with various stoichiometries. Furthermore, recent experimental evidence indicates that in hepatocytes (30) and cardiac myocytes (29), even in the presence of extracellular Na$^+$, Mg$^{2+}$ accumulation is inhibited in the absence of extracellular Ca$^{2+}$ (29) or in the presence of the Ca$^{2+}$-channel blockers verapamil (26) or nifedipine. Presently, it is also unclear whether the cellular uptake and release of Mg$^{2+}$ are performed by the same transporter or by different transporters.

Collectively, these observations indicate the presence in the plasma membrane of very active pathways for Mg$^{2+}$ transport in and out of the cell. Yet the nature of the transporters and the modality of operation are unclear. With the exception of erythrocytes, the study of Mg$^{2+}$ transport across the plasma membrane using whole cells is complicated by the fact that intracellular organelles are also active participants in cellular Mg$^{2+}$ homeostasis (32). To overcome this complication and to minimize the possible buffering of Mg$^{2+}$ by intracellular compartments in this study, sealed plasma membrane vesicles, preloaded to contain a variety of trapped ions and phosphonucleotides, were used to define the operation and selectivity of the mechanism(s) by which hepatocytes transport Mg$^{2+}$ across the cell membrane.

MATERIALS AND METHODS

Plasma Membrane Isolation

Liver plasma membrane (LPM) vesicles were isolated according to a procedure similar to that described by Prpic et...
Plasma Membrane Purity and Orientation

The purity of the LPM vesicles was assessed by using 5'-nucleotidase, cytochrome-c oxidase, and glucose-6-phosphatase activities as markers for plasma membrane, mitochondria, and endoplasmic reticulum (ER), respectively (27) (Table 1). Cytochrome-c oxidase and glucose-6-phosphatase activities were <2 nmol cytochrome c oxidized /mg protein·min⁻¹·min⁻¹ and 40 nmol P₁/mg protein·min⁻¹·min⁻¹, respectively. The orientation of 20 mM Mg²⁺-loaded and unloaded vesicles was assessed by measuring the Na⁺-K⁺-ATPase activity according to the procedure of Ismail-Beigi and Eidelberg (17), by the 5'-nucleotidase assay (27), and by [³H]ouabain binding experiments. In loaded vesicles, the Na⁺-K⁺-ATPase activity (in µmol P₁/mg protein·min⁻¹·min⁻¹) was 0.043 ± 0.006 in the absence of Triton and increased to 0.35 ± 0.082 in the presence of Triton (Table 1). When loaded vesicles were assessed for 5'-nucleotidase activity (in µmol P₁/mg protein·min⁻¹·min⁻¹) it was 0.55 ± 0.112 in the absence of Triton and 0.605 ± 0.116 in the presence of Triton. Therefore, the activities of these assays concurred in indicating that LPM were mostly in the "inside-out" configuration (86 ± 3 and 87 ± 10%, respectively). Unloaded vesicles, instead, were mainly in "inside-out" configuration, in agreement with the observation by Prpic et al. (25) (Table 1). The proper configuration in loaded vesicles is primarily due to the presence of Mg²⁺, which has been shown to significantly contribute to membrane sidedness in similar systems (36, 37). The activity of the enzymes in intact vesicles both in the presence and in the absence of ouabain was correlated with that measured in Triton X-100-treated vesicles (considered as 100%). The difference between the two activities indicates the percentage of inside-out and inside-in vesicles. Also, LPM vesicles loaded with 20 mM Mg²⁺ plus 5 mM ATP demonstrated an inside-in orientation >80%.

For the [³H]ouabain binding procedure, after the loading with Mg²⁺, LPM vesicles were incubated in the presence of 1 mM ouabain to which 50 µCi/ml [³H]ouabain was added. After 5 min of incubation, the vesicles were rapidly sedimented in microcentrifuge tubes, the supernatant was removed by vacuum suction, and the radioactivity in the pellet was measured by liquid scintillation counting. The binding occurring in intact LPM vesicles was compared with that observed in LPM vesicles permeabilized by digitonin or Triton X-100, a condition in which the maximal binding capacity of ouabain to Na⁺-K⁺-ATPase was obtained. Similarly, in [³H]ouabain binding experiments, the inside-in percentage was determined to be 72 ± 5% for 20 mM Mg²⁺-loaded vesicles. As noted in Table 1, LPM vesicles used in these studies exhibited 15% and 15.1-fold enrichment compared with homogenate in Na⁺-K⁺-ATPase and 5'-nucleotidase activity, respectively. Because the Na⁺-K⁺-ATPase is prevalent in basolateral membranes (42) whereas the 5'-nucleotidase is primarily sorted to the apical membrane of hepatocytes (35), it appears that our LPM preparation contains a similar enrichment in both of these membrane portions.

To further ascertain LPM sidedness, ⁴⁵Ca²⁺ transport experiments were performed in both unloaded and loaded vesicles, as previously described by Prpic et al. (25). Unloaded LPM, but not Mg²⁺-loaded LPM, displayed a Ca²⁺ uptake similar to that reported by Prpic et al. (25) (data not shown). In contrast, LPM loaded with 20 mM MgCl₂ plus ⁴⁵Ca²⁺ and 10 mM ATP actively extruded ⁴⁵Ca²⁺ (data not shown). Under both conditions, Ca²⁺ transport was abolished in the presence of A-23187. These data further demonstrate that loaded LPM are primarily oriented inside-out.

Endogenous carryover of cations during preparation in freshly isolated LPM was assessed for Na⁺, Mg²⁺, Ca²⁺, and K⁺ by atomic absorbance spectroscopy (AAS), using a Perkin-Elmer 3100 spectrophotometer, and found to be minimal. Negligible levels of intravesicular adenine phosphonucleotides were detected by HPLC (not shown).

Plasma Membrane Vesicle Volume and Surface Area

LPM volume and surface area were determined using two different techniques. First, LPM radius and surface area were

Table 1. Enzyme activity of purified LPM

<table>
<thead>
<tr>
<th>Fraction</th>
<th>S'-Nucleotidase</th>
<th>Mg²⁺-ATPase</th>
<th>Na⁺-K⁺-ATPase</th>
<th>Glucose-6-Phosphatase</th>
<th>Cytochrome-c Oxidase</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No Triton</td>
<td>Triton</td>
<td>No Triton</td>
<td>Triton</td>
<td>No Triton</td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.05 ± 0.009</td>
<td>0.04 ± 0.007</td>
<td>0.05 ± 0.002</td>
<td>0.06 ± 0.012</td>
<td>0.022 ± 0.007</td>
</tr>
<tr>
<td>Unloaded LPM</td>
<td>0.17 ± 0.02</td>
<td>0.324 ± 0.014</td>
<td>0.63 ± 0.27</td>
<td>0.65 ± 0.23</td>
<td>0.37 ± 0.18</td>
</tr>
<tr>
<td>Triton</td>
<td>0.550 ± 0.112</td>
<td>0.605 ± 0.116</td>
<td>0.12 ± 0.05</td>
<td>0.96 ± 0.08</td>
<td>0.043 ± 0.006</td>
</tr>
<tr>
<td>Enrichment</td>
<td>11.0</td>
<td>15.1</td>
<td>12.6</td>
<td>16.0</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 15, 8, 8, 4, and 6 for 5'-nucleotidase, Mg²⁺-ATPase, Na⁺-K⁺-ATPase, glucose-6-phosphatase, and cytochrome-c oxidase, respectively. Enzyme activities are expressed as µmol P₁/mg protein·min⁻¹·min⁻¹. Cytochrome-c oxidase activity is expressed as nmol cytochrome c oxidized/mg protein·min⁻¹·min⁻¹. All experimental protocols are described in MATERIALS AND METHODS. Enrichment was determined by comparing activity in loaded liver plasma membrane (LPM) fraction to homogenate for 5'-nucleotidase. For all other enzymatic parameters, enrichment was determined by comparing fraction with highest activity (in presence of Triton) to homogenate. ND, not determined.
determined from an electron micrograph of a typical preparation of MgCl2-loaded LPM (>9,000 magnification). The loaded LPM were sedimented through an 11% Percoll gradient and then processed for electron microscopy. The pellet was fixed overnight in 0.1 M cacodylate buffer (pH 7.4), containing 2% paraformaldehyde and 2.5% glutaraldehyde and then washed three times in 0.1 M cacodylate buffer. Postfixation occurred in 2% osmium tetroxide for 1 h, and the pellet was washed three times in double-distilled H2O. Next, the block was stained in 1% aqueous solution of uranyl acetate for 1 h, and dehydration took place in a series of graded ethanol treatments. The samples were infiltrated and embedded in Spurr low-viscosity embedding medium (Ernest F. Fullman, Latham, New York) and blocked out in Eppendorf microcentrifuge tubes. The sample was polymerized for 48 h at 70°C. The polymerized pellet block was sectioned on an ultramicrotome (Research and Manufacturing, Tucson, AZ), specifically at the center and parallel to the long axis of the Eppendorf tube, with a section thickness of 75 nm. This permitted a view of the density-separated LPM from the top to the bottom of the pellet with relative size homogeneity (larger vesicles in the bottom and smaller vesicles at the top of the pellet). Sections were mounted on Formvar-supported copper slot grids and contrasted with 2% uranyl acetate in 50% ethanol and 0.25% lead citrate in 1 N NaOH (Electron Microscopy Sciences). The section was examined and photographed in a transmission electron microscope (model J EM 1200EX II, J EOL, Peabody, MA) at 80 kV with an objective aperture of 50 µm. A transparency marked with 1 line/in. and with the lines perpendicular to the long axis of the microcentrifuge tube was placed over the micrographs. The largest 3 diameters in a field of ~25 vesicles along each line were averaged. Because the section thickness is approximately one-tenth of the diameter of the vesicles, the largest diameter along the line most closely represents the true size of the vesicles. The number of vesicles along each grid line was counted, and all the vesicles were assumed to be the size of the averaged largest diameters. A histogram of the diameters of all vesicles along the line was generated, and the data were fitted to a Gaussian function of the counted diameters. Based on these measurements and assumptions, an average diameter of 0.9 µm was determined for the entire vesicle preparation. The surface area of the LPM was calculated (Table 2) under the assumption that the vesicles were spherical in shape.

In the second technique, the intravesicular volume of LPM loaded with 20 mM Mg2+ was measured according to the procedure of Johnson and Scarpa (18) and determined as the amount of Mg2+ released into the extravesicular space and [14C]polydextran within 1 min after stimulation by 25 mM NaCl or 50 µM CaCl2; 20 mM magnesium gluconate-loaded vesicles incubated in presence of 20 mM magnesium gluconate outside were demonstrated to have largest Mg2+ efflux within 1 min after stimulation by 25 mM NaCl (344.1 ± 158.8 nmol Mg2+ /mg protein) or 50 µM CaCl2 (217.2 ± 18.2 nmol Mg2+ /mg protein). Fluxes were determined by dividing amplitude of efflux by surface area of 2.5 µm2. As discussed in text, fluxes are underestimated because sedimentation technique used does not permit initial rate measurements.

Loading of Plasma Membrane Vesicles During Isolation

In some experiments (Fig. 3), LPM vesicles were loaded during isolation, using a protocol similar to the one described above, with the following modifications. Mg2+ (10 mM) was added to the buffer before the initial homogenization and was maintained throughout the second resuspension, and the Percoll ratio was increased to 3.5 ml Percoll for every 10.4 ml resuspension. In this protocol, LPM were used immediately after isolation. An advantage of this protocol is that LPM can be simultaneously isolated and loaded.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average diameter, µm</td>
<td>0.90 ± 0.03</td>
</tr>
<tr>
<td>Total volume of membranes, µl/mg protein</td>
<td>4.90 ± 0.09</td>
</tr>
<tr>
<td>Volume per vesicle, µl/vesicle or µm3/vesicle</td>
<td>3.82 ± 10-10 or 0.382</td>
</tr>
<tr>
<td>Surface area, µm2</td>
<td>2.50</td>
</tr>
<tr>
<td>Number of vesicles per mg protein</td>
<td>1.30 × 1010</td>
</tr>
<tr>
<td>Mg2+ fluxes, nmol Mg2+·µm-2·min-1 Na+ induced</td>
<td>137.60</td>
</tr>
<tr>
<td>Ca2+ induced</td>
<td>86.80</td>
</tr>
</tbody>
</table>

Morphological calculation of diameter was determined as described in MATERIALS AND METHODS. Value represents average of 5,011 vesicles fitting to a Gaussian function with a bin range of 0.1. Intravesicular volume was determined as difference between H2O-accessible space (intravesicular and extravesicular space) and [14C]polydextran-accessible space (extravesicular space); n = 3. Details are described in MATERIALS AND METHODS. Average volume and surface area per individual vesicle were determined by assuming a spherical shape and average diameter of 0.9 µm. Number of vesicles per mg protein was calculated as ratio between average volume of vesicles per mg protein (4.9 µl/mg protein) and average volume per individual vesicle (3.82 × 10-10 µl/vesicle). Flux (nmol Mg2+·µm2·min-1) of ions across LPM per unit of surface area was determined based on efflux of Mg2+ within 1 min after stimulation by 25 mM NaCl or 50 µM CaCl2; 20 mM magnesium gluconate-loaded vesicles incubated in presence of 20 mM magnesium gluconate outside were demonstrated to have largest Mg2+ efflux within 1 min after stimulation by 25 mM NaCl (344.1 ± 158.8 nmol Mg2+ /mg protein) or 50 µM CaCl2 (217.2 ± 18.2 nmol Mg2+ /mg protein). Fluxes were determined by dividing amplitude of efflux by surface area of 2.5 µm2. As discussed in text, fluxes are underestimated because sedimentation technique used does not permit initial rate measurements.
concentration of 250 µg protein/ml. For the supernatant measurements, the loaded LPM vesicles were resuspended in Mg²⁺-free incubation medium. This was necessary because large extravesicular Mg²⁺ concentrations interfere with the measurement of Mg²⁺ content in the supernatant by AAS. For the pellet measurement, to minimize the leakage of the entrapped Mg²⁺ down its concentration gradient, the loaded LPM vesicles were resuspended in the presence (20 mM) of the cation used for the loading. In the majority of pellet experiments, the external ion concentration was 2 mM after 1:10 dilution in the incubation mixture, unless otherwise stated. In some experiments, the concentrations of intra- and extravesicular Mg²⁺ were kept identical at 20 mM.

After 1 min of equilibration, aliquots of the incubation mixture were withdrawn at 1- to 2-min intervals, and the vesicles were sedimented by rapid centrifugation (7,000 g) in microcentrifuge tubes. Mg²⁺ content was measured in the supernatants by AAS. As for the Mg²⁺ content in LPM vesicles (pellet measurement), aliquots of the incubation medium were sedimented in microcentrifuge tubes through an oil layer (dimethyl phthalate, dibutyl phthalate, and dioctyl phthalate 2:3:4) to remove extravesicular Mg²⁺. The supernatant and oil layer were removed by vacuum suction, and the pellet was digested overnight in 500 µl 10% HNO₃. The Mg²⁺ content of the vesicles was measured by AAS in the acid extracts using Mg²⁺ standards in identical acid concentration. Similar procedures were also used for Na⁺-, or Ca²⁺-loaded LPM.

Other Experimental Procedures

The intravesicular content of adenine phosphonucleotides was measured by HPLC, as described previously (5). Protein was measured according to the procedure of Bradford (1), using BSA as a standard.

Chemicals

All chemicals were of the purest analytical grade (Sigma, St. Louis, MO). [³H]louabain was from ICN (Costa Mesa, CA). All adenine and guanine nucleotides were from Calbiochem (La Jolla, CA). [³H]O₂ was obtained from Amersham, and [¹⁴C]polydextran was from Dupont-NEN. Nitrex nylon mesh was obtained from Tetko (Briarcliff Manor, NY).

Statistical Analysis

Data are presented as means ± SE. Data were first analyzed by one-way ANOVA. Multiple means were then compared by Tukey's multiple comparison test or by the Student-Newman-Keuls method performed with the level of statistical significance designated as P ≤ 0.05.

RESULTS

Electron Micrograph of Purified LPM

Figure 1 shows an electron micrograph of a typical preparation of LPM (×9,000 magnification). The contamination by other intracellular organelles is small, consistent with the biochemical determinations reported in Table 1. Based on a large body of data, the preparation of Mg²⁺-loaded LPM is between 75 and 85% “right configuration” (see Table 1 and MATERIALS AND METHODS). The average diameter, size, surface area, and number of vesicles per milligram protein were estimated in the whole population by a combination of radiochemical and morphological techniques as described in MATERIALS AND METHODS and Table 2. These values are summarized in Table 2 and were used to calculate Mg²⁺ fluxes.

Dose-Response of Na⁺- and Ca²⁺-Induced Mg²⁺ Efflux From LPM and Effects of Various Inhibitors

Figure 2, A and B, shows that LPM vesicles loaded with 20 mM MgCl₂ and suspended in a medium devoid of Na⁺ or Ca²⁺ release negligible Mg²⁺ over several minutes of incubation (control). The addition of NaCl (Fig. 2A) or CaCl₂ (Fig. 2B) to the incubation medium promotes a sizable Mg²⁺ efflux from the vesicles in a dose-dependent fashion. Irrespective of the concentration of NaCl or CaCl₂ used, Mg²⁺ efflux at 37°C is already complete within the first period of observation, 2 min after NaCl or CaCl₂ addition. The amount of Mg²⁺ released at this point is not limited by an equilibration of Mg²⁺ gradients across the plasma membrane, as additional Mg²⁺ can be mobilized from the vesicles by the addition of a divalent cation ionophore (see Fig. 5).

At 2 min, the addition of 10 or 20 mM NaCl induces the extrusion of 12.7 ± 3.9 and 37.6 ± 6.4 nmol Mg²⁺/mg protein, respectively (n = 37). The maximum Mg²⁺ extrusion is observed following the addition of 25 mM NaCl (∼90 nmol/mg protein), whereas larger concentrations of NaCl do not result in a significantly greater Mg²⁺ efflux (Fig. 2A). In addition, Fig. 2A shows that the addition of 100 mM sucrose does not induce a Mg²⁺ efflux, suggesting that the mobilization of Mg²⁺ by 50 mM NaCl is not caused by a nonspecific rapid osmotic mismatch.

Figure 2B shows that the concentrations of CaCl₂ able to induce Mg²⁺ extrusion are far lower than those of NaCl. In fact, Mg²⁺ extrusion is already detectable following the addition of 25 µM Ca²⁺ (44.9 ± 8.4 nmol Mg²⁺/mg protein) and is maximal when 500 µM CaCl₂ is added to the system (160.5 ± 11.6 nmol Mg²⁺/mg protein) (n = 8). Under these conditions, the amount of Mg²⁺ released by Ca²⁺ is almost double that mobilized by maximal concentrations of Na⁺. Higher CaCl₂ concentrations (in the millimolar range) do not result in larger Mg²⁺ efflux (not shown). Figure 2B also shows that the coaddition to LPM vesicles of 500 µM Ca²⁺ and 25 mM Na⁺, each individually producing maximal Mg²⁺ re-
lease, results in an additive amount of Mg\(^{2+}\) extruded. Specifically, the net Mg\(^{2+}\) extrusion induced by 25 mM Na\(^{+}\) plus 500 µM Ca\(^{2+}\) is 267.7 ± 17.0, compared with the Mg\(^{2+}\) efflux induced by Na\(^{+}\) or Ca\(^{2+}\) alone of 92.2 ± 13.8 and 160.5 ± 11.6 nmol Mg\(^{2+}\)/mg protein, respectively.

The results reported in Fig. 2, A and B, reflect an increase in extravesicular Mg\(^{2+}\) content (supernatant experiments). However, a quantitatively similar decrease in Mg\(^{2+}\) content retained within the vesicles could be observed in pellet experiments (see Fig. 6). Table 3 shows that the Mg\(^{2+}\) extrusion activated by Na\(^{+}\) or Ca\(^{2+}\) is decreased by 50% in the presence of 100 µM amiloride, and Ca\(^{2+}\)-induced Mg\(^{2+}\) extrusion is almost completely absent in the presence of 1 mM amiloride. Such a high concentration of amiloride has been shown to effectively inhibit Mg\(^{2+}\) extrusion via the putative Na\(^{+}\)/Mg\(^{2+}\) exchanger in several mammalian cell types, including hepatocytes (13, 38). A quantitatively similar inhibition (not shown) was also observed using 200 µM quinidine or 200 µM imipramine, two other nonspecific inhibitors of the Mg\(^{2+}\) extrusion mechanism (7). However, the coaddition of amiloride and quinidine or imipramine failed to elicit additional inhibition of the Na\(^{+}\)-dependent Mg\(^{2+}\) release (not shown). In contrast, the Ca\(^{2+}\) channel blockers nifedipine and verapamil, as well as inhibitors of the ER Ca\(^{2+}\) leak mechanism and the mitochondrial Ca\(^{2+}\) uniport, neomycin and ruthenium red, were ineffective at inhibiting Mg\(^{2+}\) extrusion from LPM (not shown).

**Table 3. Percent inhibition of Mg\(^{2+}\) efflux in LPM by amiloride**

<table>
<thead>
<tr>
<th>Amiloride Concentration, µM</th>
<th>500 µM CaCl(_2) stimulation</th>
<th>25 mM NaCl stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>39.2 ± 6.8</td>
<td>31.1 ± 1.1</td>
</tr>
<tr>
<td>100</td>
<td>58.0 ± 6.0</td>
<td>47.2 ± 14.6</td>
</tr>
<tr>
<td>500</td>
<td>78.3 ± 3.4</td>
<td>ND</td>
</tr>
<tr>
<td>1,000</td>
<td>99.9 ± 0.2</td>
<td>58.6 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE of 4 independent experiments. Aliquots of incubation mixture were withdrawn and centrifuged, and Mg\(^{2+}\) content in supernatant was measured by atomic absorbance spectrophotometry as described for Fig. 2.

**Fig. 2.** Net Mg\(^{2+}\) efflux as a function of extravesicular Na\(^{+}\) and/or Ca\(^{2+}\) concentration. Dose-response for NaCl-induced (A) or for CaCl\(_2\)-induced (B) Mg\(^{2+}\) efflux from 20 mM MgCl\(_2\)-loaded LPM vesicles, incubated in absence of extravesicular Mg\(^{2+}\). Experiments were performed as described in MATERIALS AND METHODS. Vesicles (250 µg/ml) were incubated at 37°C in a reaction medium containing 250 mM sucrose and 50 mM Tris-HEPES (pH 7.4). After 2 min of equilibration, an aliquot corresponding to 500 µl of incubation medium was withdrawn and rapidly sedimented in a microcentrifuge tube. Supernatant was assayed for Mg\(^{2+}\) content by atomic absorbance spectrophotometry (AAS). After second withdrawal, concentration of NaCl or CaCl\(_2\) indicated was added, and aliquots of sample were withdrawn in duplicate at indicated time points and processed as above. Shown is net change in Mg\(^{2+}\) content in supernatant with respect to that before ion addition. Data are means ± SE of 37 and 8 preparations for Na\(^{+}\) and Ca\(^{2+}\), respectively. ANOVA and Tukey's test for multiple comparisons or Student-Newman-Keuls method were performed at all time points. *P < 0.05 vs. control and/or 100 mM sucrose for Tukey's test; **P < 0.05 vs. control for Student-Newman-Keuls method. Additionally, in B, asterisks were omitted for clarity for 500 µM CaCl\(_2\) alone vs. 500 µM CaCl\(_2\) + 25 mM NaCl (P < 0.05).
Mg\(^{2+}\) Efflux in Preloaded Vesicles

The operation of the Na\(^{+}\)- and Ca\(^{2+}\)-dependent Mg\(^{2+}\) extrusion mechanisms was also investigated in LPM vesicles preloaded with 10 mM Mg\(^{2+}\). The major difference in this preparation is that the LPM were loaded during isolation with a concentration of 10 mM Mg\(^{2+}\), in contrast to the previous protocol in which vesicles were loaded with 20 mM MgCl\(_2\) after isolation. With this preparation, the effectiveness of CaCl\(_2\) with respect to NaCl in mobilizing Mg\(^{2+}\) is even more evident. Figure 3 shows that in these vesicles 50 µM CaCl\(_2\) prompts a Mg\(^{2+}\) extrusion similar to that reported in Fig. 2B, whereas the extrusion induced by NaCl is markedly smaller (compare Fig. 3 with Fig. 2A). Furthermore, the inhibitory effect of amiloride on the Ca\(^{2+}\)-mediated Mg\(^{2+}\) efflux is less (see Table 3).

Resolution of Mg\(^{2+}\) Kinetics by Decreasing Temperature of Incubation Medium

Figure 4 shows the temperature dependence of Mg\(^{2+}\) extrusion in supernatant experiments from 20 mM MgCl\(_2\)-loaded LPM vesicles incubated in a thermostated vessel at 15, 20, or 37°C following stimulation with 25 mM NaCl. Through a decrease in the incubation temperature to 15 or 20°C, a progressive decrease in Mg\(^{2+}\) extrusion rate is observed, and the process starts to be kinetically resolved. A qualitatively similar temperature dependence was also observed in LPM stimulated by CaCl\(_2\) (not shown).

Effects of the Ionophore A-23187

To determine the total amount of Mg\(^{2+}\) mobilizable from LPM vesicles, A-23187 (1 µg/ml) was used either in the absence of Na\(^{+}\) or after stimulation by Na\(^{+}\) (Fig. 5). As Fig. 5 shows, −35% of the entrapped Mg\(^{2+}\) is mobilized by the addition of 50 mM NaCl (Fig. 5) to the extravesicular medium. The subsequent addition of ionophore mobilizes an additional 50% of the residual intravesicular Mg\(^{2+}\) after Na\(^{+}\) stimulation. The addition of A-23187 alone rapidly mobilizes a total amount of Mg\(^{2+}\) comparable to that mobilized by Na\(^{+}\) plus the ionophore. Approximately 30% of the total Mg\(^{2+}\) is retained in LPM after addition of the ionophore. Based on the intravesicular water space, the total amount of Mg\(^{2+}\) releasable under those conditions is two- to threefold greater than the amount of “free” Mg\(^{2+}\) expected in the vesicles. This should be the result of dissociation between aspecifically bound Mg\(^{2+}\) and free Mg\(^{2+}\), as more free Mg\(^{2+}\) is released by the ionophore. Such a dissociation should, in principle, be facilitated by the intravesicular decrease in pH resulting from exchange of 1 Mg\(^{2+}\) out for 2 H\(^{+}\) in, facilitated by A-23187. Similar results were also obtained when Ca\(^{2+}\) was used instead of Na\(^{+}\) (not shown).


**TRANSPORT IN LIVER PLASMA MEMBRANE VESICLES**

Mg\(^{2+}\) Efflux Is Specific for the Monovalent Cation Na\(^{+}\)

The experiment shown in Fig. 7 was also performed under conditions of identical MgCl\(_2\) concentration inside and outside the vesicles. Figure 7 demonstrates the stringent specificity of the Na\(^{+}\)/Mg\(^{2+}\) antiporter for Na\(^{+}\). Neither Li\(^{+}\) nor K\(^{+}\) is able to elicit a Mg\(^{2+}\) efflux under these conditions.

Similarities Between Mg\(^{2+}\) and Sr\(^{2+}\) Release From Isolated LPM

Sr\(^{2+}\) has been shown to act as a substitute for Mg\(^{2+}\) in a variety of cell transport systems (29). Therefore, LPM vesicles were loaded with 20 mM Sr\(^{2+}\) (Fig. 8), and intravesicular Sr\(^{2+}\) content was measured using AAS. As Fig. 8 shows, the net loss of Sr\(^{2+}\) from the vesicles is quantitatively comparable to the net Mg\(^{2+}\) increase in the extravesicular medium (Fig. 2). For example, 25 mM NaCl and 50 µM Ca\(^{2+}\) induce an efflux of 95.9 ± 7.8 and 74.0 ± 31.3 nmol Sr\(^{2+}\)/mg protein, respectively.

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**Fig. 6.** Vesicles loaded with 20 mM magnesium gluconate were incubated in 20 mM magnesium gluconate. Shown is Mg\(^{2+}\) efflux in vesicles loaded and resuspended in presence of 20 mM magnesium gluconate. Mg\(^{2+}\) efflux is expressed as net change in Mg\(^{2+}\) content retained within LPM vs. content at time 0. Initial Mg\(^{2+}\) content before ion addition was 345.3 ± 9.8 nmol Mg\(^{2+}\)/mg protein. Data are means ± SE of 3 preparations. ANOVA and Tukey’s test for multiple comparisons were performed at all time points.* P < 0.05 vs. control.

**Mg\(^{2+}\) Transport Is Not Due to Displacement of Bound Mg\(^{2+}\)**

The experiment shown in Fig. 6 was performed under conditions in which the concentrations of MgCl\(_2\) or magnesium gluconate were identical inside and outside the vesicles (i.e., inside and outside Mg\(^{2+}\) concentrations were equal). Under these conditions, the Mg\(^{2+}\) content remaining in the pellet was measured (see MATERIALS AND METHODS). Therefore, Fig. 6 represents the amount of Mg\(^{2+}\) released from LPM as decreasing values. In vesicles loaded with magnesium gluconate, the addition of 25 mM sodium isethionate mobilizes 344.1 ± 158.8 nmol Mg\(^{2+}\)/mg protein, whereas 50 µM calcium gluconate induces an efflux of 217.1 ± 18.2 nmol Mg\(^{2+}\)/mg protein (Fig. 6) within 1 min after addition. Also, in this system, 1 mM amiloride inhibits Na\(^{+}\)- (Fig. 6) and Ca\(^{2+}\)-dependent (not shown) Mg\(^{2+}\) efflux. It is noteworthy that in the presence of 20 mM extracellular Mg\(^{2+}\) the amount of Mg\(^{2+}\) released is considerably larger than that reported in Fig. 2. Most likely, this is a consequence of a passive leakage of the cation during the loading procedure and resuspension in 0 mM Mg\(^{2+}\) (Fig. 2). Consistent with this interpretation, the amount of Mg\(^{2+}\) trapped within the vesicles resuspended in the absence of extracellular Mg\(^{2+}\) (Fig. 2) is approximately twofold less than LPM resuspended in the presence of 20 mM Mg\(^{2+}\).

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Figure 8 also shows the inhibitory effect of 200 µM imipramine on both the Na\(^{+}\) - and Ca\(^{2+}\)-dependent Mg\(^{2+}\) effluxes. Quantitatively similar data were obtained using SrCl\(_2\) labeled with \(^{85}\)Sr\(^{2+}\) (data not shown).

**Bidirectionality of Mg\(^{2+}\) Transport Mechanism(s)**

Na\(^{+}\)/Mg\(^{2+}\) transport can operate in either direction. Figure 9A shows the result of an experiment in which isolated LPM vesicles were loaded with 20 mM NaCl, as described in MATERIALS AND METHODS, and extravesicular Mg\(^{2+}\) was added at the concentrations of MgCl\(_2\) indicated in Fig. 9. Under these experimental conditions, a marked extrusion of Na\(^{+}\) from the vesicles is also observed (data not shown). Figure 9B shows that, under the same conditions, Sr\(^{2+}\) is also accumulated in a quantitatively and kinetically similar fashion, indicating that Sr\(^{2+}\) could replace Mg\(^{2+}\) for Na\(^{+}\)/Mg\(^{2+}\) exchange in either direction. The addition of Mg\(^{2+}\) or Sr\(^{2+}\) induces a large uptake that is complete within 1 min after addition.

Figure 10 shows the result of an experiment in which the effect of Mg\(^{2+}\) or Ca\(^{2+}\) was measured in Na\(^{+}\)-loaded vesicles resuspended in the absence of Na\(^{+}\). The addition of 5 mM Mg\(^{2+}\) to the extravesicular compartment prompts a net Na\(^{+}\) efflux of 235.3 ± 8.7 nmol Na\(^{+}\)/mg protein (Fig. 10), an amount approximately double that of Mg\(^{2+}\) released from Mg\(^{2+}\)-loaded vesicles stimulated by 25 mM NaCl (Fig. 2A; 92.8 ± 13.8 nmol Mg\(^{2+}\)/mg protein). In contrast, under identical conditions, 5 mM CaCl\(_2\) fails to induce Na\(^{+}\) extrusion. The small uptake of Na\(^{+}\) observable under control and Ca\(^{2+}\) conditions is likely due to the carryover of Na\(^{+}\) bound to the external face of the vesicle.

Figure 11 shows the result of experiments in which LPM vesicles were loaded with 20 mM NaCl (Fig. 11A) or with 10 mM CaCl\(_2\) (Fig. 11B) and resuspended in a medium containing the same concentration of either cation. The addition of 5 mM Mg\(^{2+}\) can induce the release of Na\(^{+}\) but not of Ca\(^{2+}\) from the vesicles, suggesting that the Na\(^{+}\)/Mg\(^{2+}\) antiporter but not the Ca\(^{2+}\)/Mg\(^{2+}\) mechanism can operate in either direction.

**Contribution of Phosphonucleotides or Phosphate Groups to Mg\(^{2+}\) Fluxes**

Several laboratories have reported the presence of an ATP-dependent Mg\(^{2+}\) transport in both mammalian (9, 21) and nonmammalian (2) cell types. HPLC determination showed that unloaded LPM vesicles contained no detectable ATP or other phosphonucleotides (not shown).
Therefore, LPM were loaded with 20 mM Mg$^{2+}$ in the absence or presence of 5 mM concentrations of different phosphonucleotides or phosphate. The results in Fig. 12 show the effects of Na$^{+}$ or Ca$^{2+}$ stimulation on Mg$^{2+}$ extrusion within 2 min after addition under a variety of phosphate loading conditions. Under all loading conditions in the absence of cation stimulation (control), LPM tightly retain trapped intravesicular Mg$^{2+}$ (Fig. 12). Both in the absence and in the presence of intravesicular ATP, the addition of Na$^{+}$ or Ca$^{2+}$ induces quantitatively similar Mg$^{2+}$ effluxes from the vesicles (Fig. 12). LPM loaded with Mg$^{2+}$ in the presence of 5 mM Tris-Pi mobilize an amount of Mg$^{2+}$ comparable to that of vesicles loaded solely with Mg$^{2+}$ after the addition of Na$^{+}$ but mobilize a much larger amount of Mg$^{2+}$ after Ca$^{2+}$ addition. Interestingly, both the Na$^{+}$- and Ca$^{2+}$-induced Mg$^{2+}$ releases are completely inhibited in vesicles loaded with 5 mM ATP$\gamma$S. Also, in the presence of ATP or P$_i$, amiloride (1 mM), quinidine, or imipramine (200 µM) inhibits both the Na$^{+}$- and Ca$^{2+}$-mediated Mg$^{2+}$ effluxes (not shown). Lastly, qualita-

Fig. 9. Influx of Mg$^{2+}$ (A) or Sr$^{2+}$ (B) into 20 mM NaCl-loaded LPM vesicles. Accumulation of Mg$^{2+}$ (A) or Sr$^{2+}$ (B) into LPM vesicles loaded with 20 mM NaCl and resuspended in presence of 2 mM NaCl in extravesicular solution. Experimental conditions were similar to those for Fig. 5. At indicated time points after addition of indicated concentrations of Mg$^{2+}$ or Sr$^{2+}$, aliquots of incubation mixture were withdrawn and sedimented through an oil layer as described in MATERIALS AND METHODS. Acid extract of pellets was assayed for Mg$^{2+}$ or Sr$^{2+}$ content by AAS. Initial Na$^{+}$ content before ion addition was 1,995.1 nmol Na$^{+}$/mg protein. One experiment typical of four similar experiments for both experimental conditions (Mg$^{2+}$ and Sr$^{2+}$) is shown.

Fig. 10. Na$^{+}$ efflux from 20 mM NaCl-loaded vesicles. Efflux of Na$^{+}$ from LPM loaded with 20 mM NaCl, incubated in absence of external NaCl, and stimulated by 5 mM Mg$^{2+}$ or 5 mM Ca$^{2+}$. Experimental conditions and Mg$^{2+}$ determinations were as for Fig. 2. Initial Na$^{+}$ content before ion addition was 2,625.4 ± 114.2 nmol Na$^{+}$/mg protein. Data are means ± SE of 3 preparations. ANOVA and Tukey’s test for multiple comparisons were performed at all time points. *P < 0.05 vs. control and 5 mM CaCl$_2$. 

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ratively similar Mg\(^{2+}\) fluxes are observed in vesicles in which ATP is replaced with an equivalent concentration of ADP (disodium or Tris salt), GTP (lithium salt), or GDP (lithium salt) (data not shown).

**DISCUSSION**

After hormonal stimulation, large fluxes of Mg\(^{2+}\) across the cell have been observed in hepatocytes (28) and other cell types (15, 23, 29, 31, 38). Depending on the signal transduction pathway activated, within 5 min of stimulation hepatocytes release or accumulate an amount of Mg\(^{2+}\) equivalent to 5–10% of their total cellular Mg\(^{2+}\), whereas cytosolic free Mg\(^{2+}\) undergoes relatively little change (28, 30). Based on the total Mg\(^{2+}\) content (15–20 mM, depending on the cell type; see Ref. 32 for a review), the amount of Mg\(^{2+}\) translocated across the plasma membrane is very large, implying the presence of transport mechanisms that are very abundant and/or operate with a rapid turnover.

During the last two decades, a large body of evidence has been obtained in molluscan (2) and in mammalian (21, 40) cells that indicates that Mg\(^{2+}\) fluxes require a physiological concentration of extracellular Na\(^{+}\) and are inhibitable by amiloride (13, 38, 40) or by other agents that affect Na\(^{+}\) transport, such as imipramine or quinidine (7). Although these studies strongly support the operation of a Na\(^{+}\)/Mg\(^{2+}\) antiporter, the requirement of additional ions, including Ca\(^{2+}\), HCO\(_3\)^\(-\), or Cl\(^{-}\), has been observed in several cell types (14, 26, 30).

**Fig. 11.** Bidirectionality of Na\(^{+}\), but not Ca\(^{2+}\), transport mechanism. Net Na\(^{+}\) or Ca\(^{2+}\) efflux was measured in vesicles loaded with 20 mM NaCl and resuspended in presence of 20 mM NaCl outside (A) or vesicles loaded with 10 mM CaCl\(_2\), and resuspended in presence of 10 mM CaCl\(_2\) outside (B). After 1 min of equilibration, 5 mM MgCl\(_2\) was added to incubation system. Na\(^{+}\) or Ca\(^{2+}\) content retained in vesicles was assayed by AAS as for Fig. 5. Initial ion contents before ion addition were 1,671.3 ± 67.5 and 585.7 ± 68.1 nmol Na\(^{+}\) or Ca\(^{2+}\)/mg protein, respectively. Data are means ± SE of 3 preparations. ANOVA and Tukey’s test for multiple comparisons were performed at all time points.* P < 0.05 vs. control.

**Fig. 12.** Loading with phosphonucleotides and phosphate moieties. Net change in Mg\(^{2+}\) content of LPM loaded with MgCl\(_2\) (20 mM) and indicated phosphonucleotides (5 mM), incubated in presence of 2 mM Mg\(^{2+}\) in extravesicular medium. Comparison of stimulatory effect on differently loaded LPM of indicated concentrations of Na\(^{+}\) or Ca\(^{2+}\) at 2 min. Amount of Mg\(^{2+}\) retained in vesicles was assayed as for Fig. 5. Initial Mg\(^{2+}\) content before ion addition was 292.5 ± 26.2 nmol Mg\(^{2+}\)/mg protein. Data are means ± SE of 10, 16, 3, and 3 preparations for control (i.e., only Mg\(^{2+}\)), ATP, Tris-Pi, and adenosine 5’-O-(3-thiotriphosphate) (ATP\(_{S}\)) loading conditions, respectively. ANOVA and Tukey’s test for multiple comparisons were performed at all time points. Stimulation of Mg\(^{2+}\) efflux by Na\(^{+}\) or Ca\(^{2+}\) is significantly different under loading with 20 mM Mg\(^{2+}\) + 5 mM ATP\(_{S}\), (P < 0.05). In presence of 20 mM Mg\(^{2+}\) + 5 mM TRIS-Pi, there is a significant difference upon stimulation by Ca\(^{2+}\) compared with other loading conditions (** P < 0.05).
These findings raise the question of the number and identity of Mg$^{2+}$ transport mechanisms in the cell plasma membrane. Furthermore, fundamental questions about the molecular identity of these transporters, their kinetics and operative parameters, and their overall regulation still await answers.

The present study was undertaken to characterize kinetically the operation of Mg$^{2+}$ transport system(s) in plasma membranes from rat hepatocytes, where Mg$^{2+}$ transport is particularly active, and to determine whether Mg$^{2+}$ uptake and/or release operates through one or several mechanisms. Such a study in intact cells is complicated by the fact that intracellular organelles play a major role in cellular Mg$^{2+}$ homeostasis (11, 32) and by the presence of intracellular ions, metabolites, and signaling or controlling machinery. Hence, a simpler system consisting of sealed, purified, and well-characterized rat LPM was used. This system has the advantage of providing single compartments in which the intravesicular milieu can be designed, modified, and controlled.

**Basic Properties of Mg$^{2+}$ Transport**

Mg$^{2+}$ extrusion and entry. Tightly sealed LPM suspended in the absence of extravascular Na$^+$ or Ca$^{2+}$ retained intravesicular Mg$^{2+}$ over a 10-min incubation period. Based on the reported values of intravesicular water space, the amount of total Mg$^{2+}$ (or Na$^+$) trapped within the vesicles is three to four times larger than that expected based on the value of the concentration of cation used for loading. This difference can be accounted for by specific binding of Mg$^{2+}$ or Na$^+$ to specific binding sites (33). The addition of Na$^+$ or Ca$^{2+}$ induces a rapid total Mg$^{2+}$ efflux from the vesicles. The amount of Mg$^{2+}$ releasable within 2 min by micromolar concentrations of Ca$^{2+}$ ranges between 30 and 50% of the total trapped Mg$^{2+}$, whereas the amount of Mg$^{2+}$ releasable by Na$^+$, at a concentration of 25 mM Na$^+$ or higher, is ~35%. These results differ from those obtained in isolated hepatocytes, in which no Mg$^{2+}$ is released in the presence of a physiological concentration of Na$^+$ or Ca$^{2+}$ in the extracellular space. In the intact hepatocyte, a smaller percentage of the total Mg$^{2+}$ is released under conditions in which cellular cAMP increases (28), whereas a Mg$^{2+}$ uptake of similar magnitude is induced by activation of protein kinase C (30). The difference in the amount of Mg$^{2+}$ mobilized from these two experimental systems does not account for differences in Mg$^{2+}$ gradients across the plasma membrane of dispersed cells or LPM and may be related to regulatory mechanisms that physiologically modulate Mg$^{2+}$ efflux in intact cells. Hence, LPM provide a system in which basic kinetic properties of Mg$^{2+}$ transport can be better defined, since plasma membrane Mg$^{2+}$ transport is effectively “uncoupled” from cellular regulatory mechanisms. Consistent with this interpretation, it is possible that the reduced effectiveness of Na$^+$ in mobilizing Mg$^{2+}$ from preloaded vesicles (Fig. 3) in comparison with LPM loaded with Mg$^{2+}$ after isolation (Fig. 2A) can be attributed to a partial retention of regulatory mechanism(s) within the vesicles, to the residual presence of cations or metabolites in the LPM, or to a different distribution of vesicle orientation. Preloaded vesicles provide a tool in which the Ca$^{2+}$-dependent mechanism can be observed independently of the Na$^+$-induced Mg$^{2+}$ extrusion. Last, because both basolateral and apical membranes are present in the LPM population (see MATERIALS AND METHODS for details), the possibilities arise that there are two distinct membrane populations rendering the distinct transport mechanisms or that the transporters are ubiquitously distributed.

Direction of Mg$^{2+}$ fluxes. Na$^+$ can induce Mg$^{2+}$ release from Mg$^{2+}$-loaded vesicles. Under appropriate conditions, this transport is fully reversible, as extravascular Mg$^{2+}$ can be accumulated in the vesicle in exchange for trapped Na$^+$. Ca$^{2+}$ can also induce Mg$^{2+}$ extrusion from Mg$^{2+}$-loaded vesicles in the absence of Na$^+$, but Mg$^{2+}$ cannot elicit an extrusion of Ca$^{2+}$ from Ca$^{2+}$-loaded vesicles (Fig. 10B). Hence, under these conditions, the operation of the Na$^+$/Mg$^{2+}$ exchanger appears to be bidirectional, whereas that of the Ca$^{2+}$/Mg$^{2+}$ exchanger is unidirectional.

The rate of Mg$^{2+}$ flux is very rapid. Within the first observation point (1 min), 344.1 ± 154.8 and 217.1 ± 18.2 nmol Mg$^{2+}$/mg protein are released following stimulation with 25 mM Na$^+$ and 50 µM Ca$^{2+}$, respectively. Under the assumption of a surface area of 2.5 µm$^2$/mg protein (Table 2), fluxes of 137.6 and 86.8 nmol Mg$^{2+}$/µm$^2$·min$^{-1}$ in exchange for Na$^+$ and Ca$^{2+}$, respectively, can be calculated. These fluxes are two to four orders of magnitude greater than those reported in intact hepatocytes for other ions (3, 34). Although very high, these fluxes are underestimated severalfold due to the inability to time resolve the initial rate.

Mg$^{2+}$ efflux is the result of true transport. Mg$^{2+}$ efflux is not the result of a passive leakage from Mg$^{2+}$-loaded vesicles, for the following reasons: 1) unstimulated LPM do not release entrapped Mg$^{2+}$ over 10 min of incubation even in the presence of a gradient across the plasma membrane (Fig. 2); 2) Na$^+$ or Ca$^{2+}$ can mobilize Mg$^{2+}$ from vesicles under conditions in which the concentrations of Mg$^{2+}$ inside and outside the vesicles are similar; and 3) both Na$^+$- and Ca$^{2+}$-induced Mg$^{2+}$ effluxes are inhibited by various agents. Mg$^{2+}$ efflux is not the result of a sudden change in osmolarity. A change in osmolarity may be significant upon addition of large Na$^+$ concentrations but is negligible upon addition of micromolar concentrations of Ca$^{2+}$. Furthermore, the addition of 100 mM sucrose (Fig. 2A), 50 mM LiCl, or 50 mM KCl (Fig. 7) fails to replace Na$^+$ or Ca$^{2+}$ in mobilizing Mg$^{2+}$.

Mg$^{2+}$ efflux is not the result of displacement of Mg$^{2+}$ loosely bound to extravascular structures, for the following reasons: 1) the rate of Mg$^{2+}$ efflux is better time resolved by decreasing the temperature of incubation; 2) both Na$^+$- and Ca$^{2+}$-stimulated Mg$^{2+}$ effluxes can still be measured in vesicles when the extra- and intravesicular Mg$^{2+}$ concentrations are equal (Fig. 6); 3) the cation ionophore A-23187 (Fig. 5) equilibrates Mg$^{2+}$ from the same pool mobilizable by Na$^+$ or Ca$^{2+}$; and 4) Mg$^{2+}$ extrusion does not occur when Na$^+$ is replaced by KCl or LiCl (Fig. 7) and is markedly
inhibited by amiloride, quinidine, or imipramine (Table 3 and Fig. 8).

Sr²⁺ can effectively replace Mg²⁺ as the transported cation. When Sr²⁺-loaded vesicles are stimulated by Na⁺ or Ca²⁺, the net Sr²⁺ efflux is quantitatively similar to the efflux of Mg²⁺ observed under similar experimental conditions. Moreover, Sr²⁺ efflux is inhibited by the same agents that inhibit Mg²⁺ fluxes. Because ²⁸Mg²⁺ is difficult to obtain and use, whereas ⁸⁵Sr²⁺ is a readily available isotope, the ability of Sr²⁺ to substitute for Mg²⁺ will provide a more useful radioactive tracer than ²⁸Mg²⁺ to monitor the movement of Mg²⁺ in different tissues, cells, or organelles.

Mg²⁺ efflux from vesicles loaded with P₁ or phosphonucleotides. The relevance of the intravesicular content of ATP for the Na⁺- and/or Ca²⁺-dependent Mg²⁺ efflux mechanisms was investigated by adding a “cytosol-like” concentration of ATP to LPM vesicles at the time of loading. The loading of LPM vesicles with 20 mM Mg²⁺ plus 5 mM ATP did not significantly modify the amplitude of Mg²⁺ extrusion induced by Na⁺ or Ca²⁺. The ineffectiveness of ATP to modify Mg²⁺ movement and the inability of 100 µM or 1 mM vanadate to affect Mg²⁺ transport (not shown) suggest that a phosphorylation event or the modulation of Mg²⁺ transport mechanism(s) via ATPases is not an absolute requirement for Mg²⁺ transport. Interestingly, the presence of ATP, both 50% enhances only the Ca²⁺-activated Mg²⁺ efflux by 50%. Taken together, these data suggest an exchange mechanism whose operation is enhanced by the presence of ATP, only when Mg²⁺ is exchanged for Ca²⁺.

Na⁺-Dependent Mechanism

Because intracellular Mg²⁺ is well below its electrochemical equilibrium in vivo (8), there is general consensus that Mg²⁺ transport across the plasma membrane could utilize the driving force of other cations, in particular Na⁺ (8). The operation of Na⁺- or Ca²⁺-dependent Mg²⁺ transport mechanisms has been reported by several laboratories (13, 21, 41) with kinetic characteristics and parameters that vary considerably among different cell types. For example, the operation of a Na⁺-/Mg²⁺ antiporter has been observed in chicken erythrocytes (13) as well as in human red blood cells (7, 21) and in rat hepatocytes (30) and cardiac myocytes (29). Yet the stoichiometry of operation of this antiporter appears to vary in different cell types. In fact, an electroneutral exchange of 2 Na⁺ in for 1 Mg²⁺ out has been reported in chicken and turkey erythrocytes (13) but not in human or other mammalian erythrocytes (21). It is also controversial whether this antiporter requires ATP to operate.

Irrespective of the exchange ratio, the Na⁺/Mg²⁺ antiporter is inhibited in intact cells by amiloride (13, 21, 38), imipramine, or quinidine (7) but not by ouabain (7), furosemide (7), bumetanide (7), 4,4'-dinitrostilbene-2,2'-disulfonic acid, or DIDS (7). Consistent with these observations, the data reported here for LPM vesicles indicate the presence of a Na⁺/Mg²⁺ exchanger that is inhibited by amiloride, imipramine, and quinidine to approximately the same degree, irrespective of different loading or stimulatory conditions. The amiloride derivatives 5-(N,N-hexamethylene)-amiloride and 5-(N,N-dimethyl)-amiloride (41), which are more specific and more effective at inhibiting other Na⁺ transport mechanisms at lower concentrations than amiloride, proved to be ineffective on LPM up to 200 µM, a finding consistent with reports by Wolf et al. (41) and by Gunzel and Schlue (16). Under our experimental conditions, 100 µM amiloride inhibits both the Na⁺- and the Ca²⁺-dependent mechanisms by 50% or more (Table 3). Because amiloride is a nonspecific inhibitor of Na⁺ transport mechanisms, its effect on Mg²⁺ fluxes may possibly be indirect, i.e., through inhibition of other transport mechanisms such as the Na⁺/H⁺ exchanger. This may explain why the degree of inhibition varies (50–90%) in our experiments under conditions in which the ionic composition of the extracellular and intravesicular milieu differs significantly. Comparing different sets of experimental data results in a variety of stoichiometric exchange ratios, suggesting the involvement of additional ions in compensating charge differential directly or indirectly.

As for the specificity of the exchanger, the addition of Na⁺ or Ca²⁺ mobilizes quantitatively similar amounts of entrapped cation from Mg²⁺- or Sr²⁺-loaded vesicles. In contrast, the addition of 5 mM Ca²⁺ results in virtually no extrusion of Na⁺ from 20 mM Na⁺-loaded LPM. This result indicates that the Na⁺/Ca²⁺ exchanger, which has negligible activity in hepatocytes (19), is not involved in the observed Mg²⁺ transport. Furthermore, the requirement for Na⁺ is very stringent because equimolar concentrations of Li⁺ or K⁺ do not induce a significant Mg²⁺ efflux from LPM (Fig. 7).

Ca²⁺-Dependent Mechanism

Several experimental reports from this (29) and other (8) laboratories indicate that Mg²⁺ transport across the plasma membranes of intact cells requires a physiological concentration of extracellular Ca²⁺. The present study suggests the presence of a Ca²⁺/Mg²⁺ exchange mechanism that operates at very low external Ca²⁺ concentrations (µM range). Compared with the Na⁺ requirement, the Ca²⁺ requirement is far less specific, since a Mg²⁺ efflux comparable to that prompted by Ca²⁺ could also be induced by addition of equimolar concentrations of other divalent cations (Ca²⁺, Mg²⁺, Mn²⁺, Sr²⁺, Ba²⁺, Cu²⁺, Cd²⁺), as preliminary experiments indicate (data not shown).

At variance with what has been observed in intact cells (29), the Ca²⁺ channel inhibitors nifedipine and verapamil are ineffective at inhibiting the extrusion of Ca²⁺-dependent Mg²⁺ efflux, whereas amiloride, imipramine, and quinidine all inhibit the process to various extents. Although the range of Ca²⁺ concentrations tested (25–500 µM) is below the physiological extracellular Ca²⁺ concentration, the Mg²⁺/Ca²⁺ exchanger appears to operate effectively at these very low concentrations. Thus it can be hypothesized that in intact
hepatocytes this system represents a constitutively active background exchanger that is very tightly controlled and that a critical controlling component(s) is lost during isolation of LPM.

At present, we have not yet identified an inhibitor able to selectively block the Ca\(^{2+}\) or Na\(^{+}\)-induced Mg\(^{2+}\) efflux. The inhibitory effect of amiloride, imipramine, and quinidine on both the Na\(^{+}\) - and the Ca\(^{2+}\)-activated effluxes could be consistent with an activation by Ca\(^{2+}\) and Na\(^{+}\) on the same Mg\(^{2+}\) transport mechanism. However, this possibility is not supported by several lines of evidence. First, the coaddition of Na\(^{+}\) and Ca\(^{2+}\) elicits an additive efflux of Mg \(^{2+}\) that is greater than that prompted by any concentration of either ion alone. Second, in preloaded vesicles, the Mg\(^{2+}\) movement elicited by Na\(^{+}\) is almost completely abolished, whereas the movement by Ca\(^{2+}\) remains quantitatively unaffected. Third, the Na\(^{+}\)/Mg\(^{2+}\) exchanger operates in either direction, whereas the Ca\(^{2+}\)/Mg\(^{2+}\) mechanism does not. These observations point to the presence and operation of two distinct mechanisms that favor the exchange of Mg\(^{2+}\) for Na\(^{+}\) and divalent cations, respectively.

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