Enhanced sodium-dependent extrusion of magnesium in mutant cells established from a mouse renal tubular cell line

Masaru Watanabe, Masato Konishi, Ichiro Ohkido, and Senya Matsufuji

1Department of Physiology, Tokyo Medical University, Tokyo, Japan; and 2Department of Biochemistry II and 3Division of Nephrology and Hypertension, The Jikei University School of Medicine, Tokyo, Japan

Submitted 7 March 2005; accepted in final form 7 May 2005

Watanabe, Masaru, Masato Konishi, Ichiro Ohkido, and Senya Matsufuji. Enhanced sodium-dependent extrusion of magnesium in mutant cells established from a mouse renal tubular cell line. Am J Physiol Renal Physiol 289: F742–F748, 2005. First published May 10, 2005; doi:10.1152/ajprenal.00091.2005.—To study the regulatory mechanisms of intracellular Mg2+ concentration ([Mg2+]i) in renal tubular cells as well as in other cell types, we established a mutant strain of mouse renal cortical tubular cells that can grow in culture media with very high extracellular Mg2+ concentrations ([Mg2+]o > 100 mM: 101Mg-tolerant cells). [Mg2+]i, was measured with a fluorescent indicator furaptra (mag-fura 2) in wild-type and 101Mg-tolerant cells. The average level of [Mg2+]i in the 101Mg-tolerant cells was kept lower than that in the wild-type cells even at 51 mM or 1 mM [Mg2+]o. When [Mg2+]i was lowered from 51 to 1 mM, the decrease in [Mg2+]i was significantly faster in the 101Mg-tolerant cells than in the wild-type cells. These differences between the 101Mg-tolerant cells and the wild-type cells were abolished in the absence of extracellular Na+ or in the presence of imipramine, a known inhibitor of Na+/Mg2+ exchange. We conclude that Na+-dependent Mg2+ transport activity is enhanced in the 101Mg-tolerant cells. The enhanced Mg2+ extrusion may prevent [Mg2+]i increase to higher levels and may be responsible for the Mg2+ tolerance.

The kidneys are extremely important for control of body Mg2+ balance through its urinary excretion, which is primarily regulated by tubular reabsorption. In the cortical thick ascending limb, Mg2+ reabsorption is thought to occur by passive transport through the paracellular pathway. On the other hand, Mg2+ reabsorption in distal tubules is transcellular and active; Mg2+ enters into the tubular cells through the apical membrane by passive influx driven by the electrochemical gradient possibly through some transient receptor potential (TRP) channels and extruded from the cells through the basolateral membrane by active transport. (for review, see Refs. 1 and 12). Extracellular Na+-dependent Mg2+ extrusion (the putative Na+/Mg2+ exchange) has been postulated as the active transport mechanism in renal tubular cells, as well as in other cell types (for reviews, see Refs. 5 and 15). However, the transporter molecules are not yet identified, hindering detailed characterization of the transport. A cultured cell line that highly expresses any target molecule, if established, could be a useful tool for molecular cloning. In the present study, as the first step toward molecular cloning of the Na+/Mg2+ exchanger, we established a mutant cell line from mouse renal cortical tubular (MCT) cells (9) that could grow in culture media with very high extracellular Mg2+ concentration ([Mg2+]o > 100 mM: 101Mg-tolerant cells) by stepwise increases of Mg2+ concentration in the culture media and selection of high-Mg2+-tolerant cells. Intracellular Mg2+ concentration ([Mg2+]i) was measured with a fluorescent indicator furaptra (mag-fura 2) in 101Mg-tolerant and the wild-type cells, and the characteristics of their Mg2+-extruding activities were compared.

Some of these results have been published previously in abstract form (22).

MATERIALS AND METHODS

Cell culture. A line of mouse cortical tubular epithelial cells, MCT cells (9), was kindly provided by Drs. I. Inoue and J. M. Lalouel of the University of Utah. The cells were maintained in DMEM (Invitrogen, Tokyo, Japan) supplemented with 5% FCS (Invitrogen) under 10% CO2-air at 37°C. For passage, cells were washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 20 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) followed by dissociation with 0.25% trypsin (In vitrogen). Compositions of media used for establishing Mg2+-tolerant cells are listed in Table 1. They were made up by mixing the vitamin mixture (Invitrogen) and other stock solutions prepared from individual chemicals. Osmolality of the culture media was measured by the freezing-point method using an osmometer (Osmotron-20; Orion, Tokyo, Japan).

Microscopy. Cells grown on a culture dish (Corning, Corning, NY) were observed and photographed with an inverted phase-contrast microscope (Olympus, Tokyo, Japan) equipped with a digital camera (PDMC IC, Polaroid, Waltham, MA). For electron microscopy, cells on a culture dish were washed twice with PBS and fixed in 2% glutaraldehyde in 0.1 M Na3HPO4, pH 7.4 followed by dissociation with 0.25% trypsin (Invitrogen). Compositions of media used for establishing Mg2+-tolerant cells are listed in Table 1. They were made up by mixing the vitamin mixture (Invitrogen) and other stock solutions prepared from individual chemicals. Osmolality of the culture media was measured by the freezing-point method using an osmometer (Osmotron-20; Orion, Tokyo, Japan).

Optical measurements. Either 101Mg-tolerant or wild-type cells were grown on glass-bottomed culture dishes (Matsunami Glass, Osaka, Japan) and placed on the stage of an inverted microscope (TE300, Nikon, Tokyo, Japan). Apparatus, methods for fluorescence measurements, and analyses have been described previously (19). Briefly, cell clusters in a 300-μm-diameter field were alternately illuminated with light beams of 350 nm (an isosbestic wavelength for Mg2+) and 382 nm (Mg2+-sensitive wavelength) through a ×40 objective (CFI S Fluor40, Nikon). A shutter for the excitation light beam was opened for 10 s, and emitted fluorescence at 500 nm [25 nm full width at half-maximum (FWHM)] at each excitation wavelength was collected by a photomultiplier tube (H7500, Hitachi, Tokyo, Japan).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Table 1. Composition of culture media

<table>
<thead>
<tr>
<th>Medium*</th>
<th>MgCl₂</th>
<th>MgSO₄</th>
<th>NaCl</th>
<th>Dextran</th>
<th>Osmolality, mM osmol/kg H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1 mM Mg²⁺</td>
<td>0</td>
<td>0.8</td>
<td>110</td>
<td>0</td>
<td>284</td>
</tr>
<tr>
<td>+61 mM Mg²⁺</td>
<td>60</td>
<td>0.8</td>
<td>50</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>+71 mM Mg²⁺</td>
<td>70</td>
<td>0.8</td>
<td>30</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>+81 mM Mg²⁺</td>
<td>80</td>
<td>0.8</td>
<td>30</td>
<td>0</td>
<td>353</td>
</tr>
<tr>
<td>+91 mM Mg²⁺</td>
<td>90</td>
<td>0.8</td>
<td>30</td>
<td>0</td>
<td>383</td>
</tr>
<tr>
<td>+101 mM Mg²⁺</td>
<td>100</td>
<td>0.8</td>
<td>30</td>
<td>0</td>
<td>403</td>
</tr>
<tr>
<td>+1 mM Mg²⁺</td>
<td>1</td>
<td>0.8</td>
<td>110</td>
<td>20</td>
<td>400</td>
</tr>
</tbody>
</table>

*The media also contain 5% FCS, 1 g/l D-glucose, and other components of DMEM as originally indicated (3). †Osmolality was directly measured as described in MATERIALS AND METHODS. ND, not determined.

After measurement of the background fluorescence from the cell cluster within the optical field, cells were incubated with 5 μM AM ester of furaptra for 12 min at room temperature. The AM ester was then washed off for at least 10 min by continuous flow of the perfusate. The background fluorescence was subtracted from the total fluorescence measured after the indicator loading to calculate indicator fluorescence intensities with excitation at 350 nm (F₃₅₀) and 382 nm (F₃₈₂).

Because the instability of optical components caused small drifts during the study, we occasionally measured the ratio of F₃₈₂/F₃₆₀ in a Ca²⁺- and Mg²⁺-free buffer solution (140 mM KCl, 10 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 10 mM PIPES, pH 7.1) filled in thin-wall quartz capillaries (internal diameter of ~50 μm) as a standard. All values of F₃₈₂/F₃₅₀ measured from cells were normalized to the standard F₃₈₂/F₃₅₀ value, and the normalized F₃₈₂/F₃₅₀ was converted to [Mg²⁺], with the equation

\[ [Mg^{2+}] = K_D \cdot \frac{R - R_{min}}{R_{max} - R} \]  

where R_{min} and R_{max} are the F₃₈₂/F₃₅₀ values at zero [Mg²⁺] and saturating [Mg²⁺], respectively. We used parameter values previously estimated in cardiac myocytes [R_{min} of 0.969, R_{max} of 0.223, and K_D of 5.30 mM (21)] because intracellular calibration of furaptra fluorescence signals was not successful in the MCT cells, owing to significant leakage of the indicator (~20% in 20 min).

In some experiments, fura 2 was introduced by incubation with fura 2-AM for 20 min at room temperature. After background subtraction, F₃₈₂/F₃₅₀ of fura 2 was normalized to the standard F₃₈₂/F₃₅₀ measured in the Ca²⁺- and Mg²⁺-free solution (above) and used as the Ca²⁺-related signal. Because the present purpose was to compare relative changes in intracellular Ca²⁺ concentration ([Ca²⁺]i) in the 101Mg-tolerant and wild-type cells, no attempt was made to calibrate fura 2 F₃₈₂/F₃₅₀ in terms of [Ca²⁺]i.

Solutions and chemicals. Measurements of [Mg²⁺], were carried out in Ca²⁺-free solutions containing 0.5 mM EGTA to minimize any interference by Ca²⁺-related fluorescence change of furaptra (8, 18, 19). The normal-Mg²⁺ solution contained (in mM) 150 NaCl, 4 KCl, 0.5 EGTA, 1 Mg(methanesulfonate), 10 glucose, and 5 HEPEs, with pH adjusted with Tris·HCl to 7.40 at 25°C. The high-Mg²⁺ solution contained 50 mM MgCl₂ with N-acetylglucosamine reduced to 75 mM to maintain the osmolality constant at 310 mosmol/kg H₂O. In some experiments, Mg²⁺ concentration was further increased to 101 mM, while Na⁺ was eliminated. Low-Na⁺ solutions were prepared by equimolar substitution of Na⁺ with N-methyl-d-glucamine. For measurements of Ca²⁺-related signals of fura 2, Ca²⁺ concentration of the solutions was raised by replacement of 0.5 mM EGTA with 2 mM CaCl₂. Furaptra-AM (mag-fura 2-AM), furaptra (mag-fura 2, 4 K⁺ salt), fura 2-AM, and fura 2 (5 K⁺ salt) were purchased from Molecular Probes (Eugene, OR). Dextran (T-40, molecular weight of 36,000–43,000) was purchased from Amersham (Piscataway, NJ). All other chemicals were reagent grade.

Data analysis. Nonlinear and linear least-square fittings were carried out with the program Kaleidograph (version 3.501; Synergy Software, Reading, PA). The two-tailed Student’s t-test was used for statistical comparison with the significance level set at P < 0.05, unless otherwise noted. Statistical values were given as means ± SD.

RESULTS

Establishment of Mg-tolerant MCT cells. The standard culture medium for MCT cell culture was replaced by media containing various concentrations of Mg²⁺ (Table 1) and incubated replacing the medium with fresh media twice per week. In a medium containing 41 mM or a lower concentration of Mg²⁺, cells showed no apparent changes in their shape and growth rate. However, ~80% of the cells in the 61 mM Mg²⁺-containing medium (Mg-61mM) and all of the cells in 81 mM or higher Mg²⁺-containing medium died within 7 days. In contrast, addition of 20% dextran (molecular weight of ~40,000) to the 1 mM Mg medium (osmolality of 400 mosmol/kg H₂O) did not affect the growth capability of the cells (unpublished data), indicating that high-Mg²⁺ concentration rather than high osmolality (353 mosmol/kg H₂O) of the 81 mM Mg medium caused cell extinction. The surviving cells in Mg-61mM medium started dividing thereafter, and the cells reached confluence 21 days after increasing Mg²⁺ concentration.

At this time point, the cells were dissociated with trypsin and diluted 10-fold into a fresh Mg-61mM medium. After passages in the Mg-61mM medium for 6 wk (i.e., dissociation and 10-fold dilution when cells reached confluence), the culture medium was changed to one containing 71 mM Mg. Thereafter, the Mg²⁺ concentration of the medium was further increased every 10–12 wk in increments of 10 mM. Massive cell death was observed when Mg²⁺ concentration was elevated from 71 to 81 mM and from 81 to 91 mM. The cells that had adapted to 81 and 101 mM Mg²⁺ could be dislodged and stored in 10% DMSO and 90% FCS in liquid nitrogen, with 20–50% of viability at retrieval. MCT cells could be adapted in DMEM containing as much as 121 mM Mg²⁺ without losing their growing capacity.

Characterizations of the Mg-tolerant cells. The 101Mg-tolerant cells grew at a significantly slower rate than the nontolerant (wild-type) cells. The approximate doubling time was 40 h for the 101Mg-tolerant cells and 13 h for the wild-type cells. The 101Mg-tolerant cells also showed a distinct morphology. Under a phase-contrast microscope, growing wild-type cells formed islet-like groups of monolayer cells, and each cell seemed to attach tightly. In contrast, the 101Mg-tolerant cells grew without forming groups, and cell-to-cell contact was very rough (Fig. 1). Transmission electron microscopy, growing wild-type cells formed islet-like groups of monolayer cells, and each cell seemed to attach tightly. Desmosomes were often observed at the contact point of the 101Mg-tolerant cells, suggesting that nondesmosomal cellular attachment is impaired in these cells.

Effect of extracellular Mg²⁺ and Na⁺ on [Mg²⁺]. Figure 2 summarizes the effects of [Mg²⁺], and extracellular Na⁺ concentration ([Na⁺]), on [Mg²⁺] in the 101Mg-tolerant and wild-type MCT cells. In the presence of 150 mM Na⁺, [Mg²⁺], in the 101Mg-tolerant cells was significantly lower than that in...
the wild-type cells at 1 mM and also 51 mM \([\text{Mg}^{2+}]_o\). In the absence of \([\text{Na}^+]_o\), however, there was no significant difference in \([\text{Mg}^{2+}]_i\) between the 101Mg-tolerant and wild-type cells. These results suggest that \([\text{Mg}^{2+}]_i\) of the 101Mg-tolerant cells is lowered by an extracellular \(\text{Na}^+\)-dependent mechanism.

Comparison of the \(\text{Mg}^{2+}\) extrusion activity in the 101Mg-tolerant and the wild-type MCT cells. To evaluate the rate of \(\text{Mg}^{2+}\) extrusion in the 101Mg-tolerant and wild-type cells, time-resolved measurements of \([\text{Mg}^{2+}]_i\) were carried out on reduction of \([\text{Mg}^{2+}]_o\) from 51 to 1 mM. In the presence of 150 mM \(\text{Na}^+\), reduction of \([\text{Mg}^{2+}]_o\) to 1 mM caused a decrease in \([\text{Mg}^{2+}]_i\), with the average rate much greater in the 101Mg-tolerant cells than in the wild-type cells (Fig. 3). The decrease in \([\text{Mg}^{2+}]_i\) was largely diminished in the absence of extracellular \(\text{Na}^+\) in both cell types, indicating the \(\text{Na}^+\)-dependent \(\text{Mg}^{2+}\) extrusion activity.

For quantitative comparison of the rate of \(\text{Mg}^{2+}\) extrusion in the 101Mg-tolerant and wild-type cells, we analyzed changes in \([\text{Mg}^{2+}]_i\) during the initial 7 min after reduction of \([\text{Mg}^{2+}]_o\) (\(\Delta[\text{Mg}^{2+}]_i\)). The interval of 7 min was chosen because the greatest changes in \([\text{Mg}^{2+}]_i\) occurred during this period in the

Fig. 1. A: inverted phase-contrast microscopy of wild-type mouse renal cortical tubular (MCT) cells. B: cells in high extracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_o\) >100 mM: 101Mg-tolerant cells). Bars = 50 \(\mu\)m. C: electron microscopy of wild-type MCT cells. D: 101Mg-tolerant cells. Bars = 1 \(\mu\)m.

Fig. 2. Effects of extracellular Mg\(^{2+}\) and Na\(^+\) concentrations ([Mg\(^{2+}\)]\(_o\) and [Na\(^+\)]\(_o\), respectively) on levels of intracellular Mg\(^{2+}\) concentration ([Mg\(^{2+}\)]\(_i\)). Each pair of columns compared mean (±SD, \(n = 8–12\)) values of [Mg\(^{2+}\)]\(_i\) in the 101Mg-tolerant cells (open columns) and the wild-type cells (filled columns) measured after the cells had been incubated for 40 min in the solutions of [Mg\(^{2+}\)]\(_o\) and [Na\(^+\)]\(_o\), indicated. *\(P < 0.01\).
101Mg-tolerant cells (Fig. 3). In the presence of 150 mM Na\(^{+}\), \(\Delta[Mg^{2+}]\) was found to be linearly related to initial \([Mg^{2+}]\); for both the 101Mg-tolerant cells and wild-type cells (Fig. 4A); values of \(\Delta[Mg^{2+}]\) were more negative for the higher initial \([Mg^{2+}]\). However, the slope of the relation was clearly steeper, and the decrease in the \([Mg^{2+}]\) was faster at any given initial \([Mg^{2+}]\), for the 101Mg-tolerant cells than for the wild-type cells (Fig. 4A). On the other hand, in the absence of extracellular Na\(^{+}\), \(\Delta[Mg^{2+}]\) values were close to zero independent of initial \([Mg^{2+}]\), in both cell types (Fig. 4B). These results suggest the existence of a Na\(^{+}\)-dependent Mg\(^{2+}\) extrusion mechanism in the MCT cells and enhancement of its activity in the cells of acquired tolerance to very high \([Mg^{2+}]\).

The rate of Mg\(^{2+}\) influx was also estimated in the 101Mg-tolerant and the wild-type cells by time-resolved measurements of \([Mg^{2+}]_{i}\), carried out after elevation of \([Mg^{2+}]_{o}\), from 1 to 51 mM to facilitate Mg\(^{2+}\) influx. The solution of 51 mM \([Mg^{2+}]_{o}\) did not contain Na\(^{+}\) to minimize Mg\(^{2+}\) efflux. In 7 min, \([Mg^{2+}]_{i}\) slightly rose from 0.379 \pm 0.161 to 0.472 \pm 0.190 mM (\(n = 3\)) in the 101Mg-tolerant cells and from 0.752 \pm 0.186 to 0.856 \pm 0.260 mM (\(n = 3\)) in the wild-type cells; the \([Mg^{2+}]_{i}\) increments in the 101Mg-tolerant and the wild-type cells were not significantly different. Thus the rate of Mg\(^{2+}\) influx appears to be similar in the 101Mg-tolerant cells and the wild-type cells.

Extracellular Na\(^{+}\) dependence. To analyze Na\(^{+}\) dependence of the Mg\(^{2+}\) influx, we measured \(\Delta[Mg^{2+}]\) in the 101Mg-tolerant cells at various \([Na^{+}]_{o}\). For this purpose, we selected cell clusters with a similar initial \([Mg^{2+}]\), of \(-0.9\) mM (0.91 \pm 0.13 mM, \(n = 24\)). The results of a series of experiments carried out with the same subculture on the same day clearly showed that \([Na^{+}]_{o}\), accelerated, in a concentration-dependent manner, the decrease in \([Mg^{2+}]_{i}\) induced by reduction of \([Mg^{2+}]_{o}\) from 51 to 1 mM (Fig. 5A). The data thus obtained...

---

**Fig. 3.** Changes in \([Mg^{2+}]\), in the wild-type cells (A) and the 101Mg-tolerant cells (B) after reduction of \([Mg^{2+}]\), from 51 to 1 mM. After cells had been incubated for 40 min in the high-Mg\(^{2+}\) solution containing 51 mM Mg\(^{2+}\) and 75 mM Na\(^{+}\), the perfusate was quickly changed at "time 0" to the normal Mg\(^{2+}\) solution that contained 1 mM Mg\(^{2+}\) plus either 150 mM Na\(^{+}\) (○, ●) or 0 mM Na\(^{+}\) (●, ○). Each point represents mean \(\pm SD (n = 11)\).

**Fig. 4.** Analyses of the rate of change in \([Mg^{2+}]\) (\(\Delta[Mg^{2+}]\)) at 150 mM Na\(^{+}\) (A) and 0 mM Na\(^{+}\) (B) obtained from the type of experiments shown in Fig. 3. Data obtained from individual cell clusters of the 101Mg-tolerant cells (○, ●) and the wild-type cells (○, ●) are compared. Changes in \([Mg^{2+}]\), during 7 min after reduction of \([Mg^{2+}]\), to 1 mM are plotted against initial levels of \([Mg^{2+}]\) at 51 mM \([Mg^{2+}]_{o}\). Each symbol represents data obtained from a different cell cluster, and the regression line is also shown for each data set. Three data points with asterisks in A were measured after cells had been incubated in 101 mM \([Mg^{2+}]_{i}\) for 40 min to raise initial \([Mg^{2+}]_{i}\) to higher levels.

**Fig. 5.** Effect of \([Na^{+}]_{o}\) on \(\Delta[Mg^{2+}]\), in the 101Mg-tolerant cells. A: results from a series of experiments carried out on the same subculture. \([Mg^{2+}]_{o}\), was reduced from 51 to 1 mM at "time 0" in the presence of various Na\(^{+}\) concentrations: 150 mM (●), 75 mM (○), 37.5 mM (△), 18.7 mM (▲), 9.37 mM (■) and 0 mM (□). Each symbol represents data obtained from a different cell cluster. B: \(\Delta[Mg^{2+}]\), values measured in 6 series of experiments were plotted as a function of \([Na^{+}]_{o}\). Each symbol represents mean \(\pm SD\) of 4 cell clusters. A smooth line indicates the least squares fit by the Hill-type curve with parameters shown in the panel: \(\Delta[Mg^{2+}]_{i} = \min + \max \times \frac{[Na^{+}]_{o}^{n} - \min}{\max - \min} \times \frac{[Na^{+}]_{o}^{n}}{[Na^{+}]_{o}^{n} + K_{1/2}^{n} + N} \), where \(n\) and \(\min\) and \(\max\) denote, respectively, \(\Delta[Mg^{2+}]_{i}\), values at zero and saturating \([Na^{+}]_{o}\), \(N\) is the Hill coefficient, and \(K_{1/2}\) is \([Na^{+}]_{o}\), that gives a midpoint value of \(\Delta[Mg^{2+}]_{i}\) between \(\min\) and \(\max\).
from a total of 24 cell clusters were explained by the Hill-type curve with a Hill coefficient of ~2 and half activation at 25 mM [Na\(^++\)]\(_{i}\). (Fig. 5B).

**Effect of imipramine.** We also examined the effects of imipramine, a known inhibitor of the Na\(^+\)/Mg\(^2+\) exchange in erythrocytes (4, 6) and cardiac myocytes (8, 17), on the rate of decrease in [Mg\(^2+\)]\(_{i}\) at 150 mM [Na\(^++\)]\(_{o}\). In the selected clusters of the 101Mg-tolerant cells that had similar initial [Mg\(^2+\)]\(_{i}\) of \(\sim 0.8\) mM (0.813 \(\pm\) 0.106 mM, \(n = 20\)), imipramine slowed decreased [Mg\(^2+\)]\(_{i}\) in a concentration-dependent manner (Fig. 6); \(\Delta[Mg^{2+}]_{i}\) in the presence of 200 \(\mu\)M imipramine was not significantly different from that in the absence of extracellular Na\(^+\) (Scheffé's post hoc analysis; \(P > 0.05\)). Thus imipramine appears to inhibit most, if not all, of the extracellular Na\(^+\)-dependent Mg\(^2+\) extrusion activity with a half-inhibitory concentration between 50 and 200 \(\mu\)M.

**Comparison of Na\(^+\)/Ca\(^2+\) exchange activity in the Mg\(^2+\)-tolerant and the wild-type MCT cells.** Because it has been reported that the Na\(^+\)/Ca\(^2+\) exchanger can transport Mg\(^2+\) and may play a role in the extrusion of Mg\(^2+\) from cells (18), we compared Na\(^+\)/Ca\(^2+\) exchange activity in the 101Mg-tolerant cells with that in the wild-type cells by monitoring fura 2 fluorescence signals. In the presence of extracellular 2 mM Ca\(^2+\), withdrawal of extracellular Na\(^+\) induced a slight decrease in fura 2 F\(_{382}/F_{360}\), as expected for the small elevation of [Ca\(^2+\)]\(_{i}\), and F\(_{382}/F_{360}\) rapidly returned to the base level after reinduction of Na\(^+\) (Fig. 7). In the 101Mg-tolerant and wild-type cells, Na\(^+\) withdrawal caused changes of fura 2 F\(_{382}/F_{360}\) in 2 min by \(-0.050 \pm 0.0015\) (\(n = 4\)) and \(-0.058 \pm 0.022\) (\(n = 5\)), respectively, from the initial levels of 0.815 \(\pm\) 0.023 and 0.725 \(\pm\) 0.058, respectively. These Na\(^+\)-free-induced changes in F\(_{382}/F_{360}\) in the 101Mg-tolerant cells and the wild-type cells were not significantly different, suggesting similarly low activity of the Na\(^+\)/Ca\(^2+\) exchanger in both cell types. Low activity of the Na\(^+\)/Ca\(^2+\) exchanger was consistent with low expression levels of mRNA; DNA microarray analysis indicated that mRNA levels for NCX1 (a major isof orm known to be expressed in kidney; e.g., 13) were under the detection threshold in both 101Mg-tolerant cells and wild-type cells (data not shown).

**DISCUSSION**

**General.** We successfully established high-Mg\(^2+\)-tolerant MCT cells, probably through genetic change(s) in the cellular genome due to selection under high-Mg\(^2+\) conditions. The implications of the morphological changes in the high-Mg\(^2+\)-tolerant cells (Fig. 1) are not known at this point. In the present study, we focused on the functional differences in Mg\(^2+\) homeostasis of the 101Mg-tolerant and wild-type cells.

Quantitative measurements of [Mg\(^2+\)]\(_{i}\) require calibration of furaptra F\(_{382}/F_{360}\) in the cell interior (intracellular calibration), since properties of furaptra are likely altered in the cytoplasm (16, 19) probably as a result of the indicator binding to cellular proteins. Although we used parameter values previously estimated in cardiac myocytes (\(R_{\text{min}}\) of 0.969, \(R_{\text{max}}\) of 0.223, \(K_d\) of 5.30 mM), similar parameter values were also obtained for furaptra in tenia cecum: \(R_{\text{min}}\) of 0.986, \(R_{\text{max}}\) of 0.199, and \(K_d\) of 5.43 mM (16). Thus it appears that intracellular properties of the indicator are similar in different cell types and may cover the potential differences in the calibrations for the MCT cells vs. cardiac myocytes. Note, however, that values of \(R_{\text{max}}\) and \(R_{\text{min}}\) are instrument dependent and must be determined in each system. It has been reported that neither addition of imipramine (up to 200 \(\mu\)M) nor equimolar substitution of K\(^+\) by Na\(^+\) (up to 20 mM) markedly affects furaptra F\(_{382}/F_{360}\) in the solutions containing 0–4 mM Mg\(^2+\) (16).

Because the extracellular Na\(^+\) dependence and imipramine sensitivity found in the present study are similar to those of the Na\(^+\)/Mg\(^2+\) exchange reported in other cell types with different techniques (see below), most of the changes in [Mg\(^2+\)]\(_{i}\), observed in the present experimental conditions likely reflect Mg\(^2+\) transport across the cell membrane, rather than alterations in intracellular Mg\(^2+\) buffering and sequestration by organelles. In Ca\(^2+\)-free conditions where [Ca\(^2+\)]\(_{i}\) does not change significantly, competition of binding sites between Ca\(^2+\) and Mg\(^2+\) is probably minimized, and Mg\(^2+\) fluxes between the cytoplasm and mitochondria are also suppressed (2). Although we used N-methyl-D-glucamine to replace Na\(^+\) for low-Na\(^+\) solutions in the present study, we previously reported that the rates of extracellular Na\(^+\)-dependent Mg\(^2+\) efflux were essentially unaffected by use of tetramethylammonium to replace Na\(^+\) in cardiac myocytes (19).
Acquisition of Mg\(^{2+}\) tolerance. The increase in Mg\(^{2+}\) concentration accompanied the reduction of Na\(^{+}\) concentration in the culture media (Table 1). It is unlikely, however, that low-Na\(^{+}\) concentration plays a principal role in the prevention of cell growth because massive cell death was observed when Mg\(^{2+}\) concentration was elevated from 71 to 81 mM and from 81 to 91 mM (see above), whereas Na\(^{+}\) concentration was kept constant at 30 mM (Table 1). It is possible, however, that low Na\(^{+}\) concentration may facilitate Mg\(^{2+}\) overloading of the cells by partial inhibition of the Na\(^{+}\)-dependent Mg\(^{2+}\) extrusion (Fig. 5).

The mechanism responsible for low [Mg\(^{2+}\)], in the Mg\(^{2+}\)-tolerant cells could be attributed to 1) decreased Mg\(^{2+}\) influx, 2) increased Mg\(^{2+}\) extrusion, and 3) other intracellular changes. The subsequent kinetic study of [Mg\(^{2+}\)], by the fluorescent indicator suggests that point 2, above, is the most likely mechanism. Mg\(^{2+}\) influx is likely mediated by Mg\(^{2+}\)-permeable TRP channels, such as TRP-M6 and TRPM7, which can permeate Mg\(^{2+}\) either in the presence or in the absence of extracellular Na\(^{+}\) (11). Suppression of this Na\(^{+}\)-dependent Mg\(^{2+}\) influx pathway could lead to Mg\(^{2+}\) tolerance. However, the rate of Mg\(^{2+}\) influx (measured at 51 mM [Mg\(^{2+}\)], above) appears to be similar in the 101Mg-tolerant cells and the wild-type cells and is probably much slower at 1 mM [Mg\(^{2+}\)], than that of Mg\(^{2+}\) efflux. Alternatively, facilitation of the Na\(^{+}\)-independent Mg\(^{2+}\) efflux through the TRP channels, if it occurs under condition of a reversed electrochemical gradient of Mg\(^{2+}\), could play a role in the acquisition of Mg\(^{2+}\) tolerance. It should be noted, however, that Mg\(^{2+}\) extrusion in the absence of extracellular Na\(^{+}\) (possibility via the Na\(^{+}\)-independent passive pathway) was similar in the 101Mg-tolerant and wild-type cells and was very slow even in the absence of extracellular Ca\(^{2+}\), in which Mg\(^{2+}\) permeation through the channels was enhanced (Fig. 4B). Thus suppression of passive Mg\(^{2+}\) influx or enhancement of passive Mg\(^{2+}\) extrusion, if any, does not seem to explain the difference between the 101Mg-tolerant cells and the wild-type cells.

Changes in buffering and sequestration of intracellular Mg\(^{2+}\) do not seem to be reconcilable with the observed effects of extracellular Na\(^{+}\) and imipramine, unless these mechanisms are highly dependent on Na\(^{+}\) and imipramine; Δ[Mg\(^{2+}\)], of the 101Mg-tolerant cells was markedly reduced in the absence of extracellular Na\(^{+}\) or in the presence of imipramine (200 μM) to the levels similar to those observed in the wild-type cells (Fig. 6).

The average levels of basal [Mg\(^{2+}\)], estimated at normal [Mg\(^{2+}\)], of ~1 mM were in the submillimolar range in both the 101Mg-tolerant and wild-type cells, as reported with various methods in a number of different cell types (for review, see Ref. 14). In the high [Mg\(^{2+}\)], conditions (51 mM), the average [Mg\(^{2+}\)] of the wild-type MCT cells was increased above 1.0 mM, whereas that of the 101Mg-tolerant cells remained lower than 1.0 mM in the presence of extracellular Na\(^{+}\). The lower [Mg\(^{2+}\)], found in the 101Mg-tolerant cells probably accounts for, at least in part, their acquisition of high-Mg\(^{2+}\) tolerance. However, it is also possible that there are other changes in intracellular Mg\(^{2+}\) metabolism (i.e., intracellular binding and sequestration) in the 101Mg-tolerant cells that makes the cells resistant to high [Mg\(^{2+}\)]. It should also be noted that some of genetic changes in the 101Mg-tolerant cells could result from, rather than cause, the Mg\(^{2+}\) tolerance.

Further studies are required to determine the precise mechanisms of the Mg\(^{2+}\) tolerance.

Enhanced Mg\(^{2+}\) extrusion in the 101Mg-tolerant cells. The present results clearly indicate that the Na\(^{+}\)-dependent net Mg\(^{2+}\) efflux can significantly lower [Mg\(^{2+}\)], of the 101Mg-tolerant cells within several minutes. Because influx of Mg\(^{2+}\) appears to be rate limited by low permeability of the cell membrane for Mg\(^{2+}\), the enhanced net Mg\(^{2+}\) efflux in the 101Mg-tolerant cells observed in the present study likely reflects, for the most part, the active Mg\(^{2+}\) extrusion activity. The contribution of the Na\(^{+}/Ca\(^{2+}\) exchanger on the enhanced Mg\(^{2+}\) extrusion is probably, if any, minor, because the extracellular Na\(^{+}\)-dependent changes in [Ca\(^{2+}\)], (as judged from fura 2 fluorescence) appear to be similar in the 101Mg-tolerant and the wild-type cells (Fig. 7).

The Mg\(^{2+}\) extrusion from the 101Mg-tolerant cells had a K\(_{1/2}\) value for Na\(^{+}\) of 25 mM; i.e., the transport was half-activated at 25 mM [Na\(^{+}\)] (Fig. 5). This K\(_{1/2}\) value is similar to those reported in chicken erythrocytes (25 mM; Ref. 7), membrane vesicles from rabbit ileum (16 mM; Ref. 10), and smooth muscle of guinea pig tenia cecum (27 mM; Ref. 16). Thus the extracellular Na\(^{+}\) dependence of Mg\(^{2+}\) extrusion observed in the present study is consistent with those previously reported, although different estimates for K\(_{1/2}\) have also been reported in different cell types (for review, see Refs. 5 and 15). The Hill coefficient of ~2 for the relation between [Na\(^{+}\)], and the rate of Mg\(^{2+}\) extrusion (Fig. 5) could be explained by Na\(^{+}/Mg\(^{2+}\) exchange with a stoichiometry of 2:1. However, further studies are necessary to establish the stoichiometry of the transport.

Imipramine inhibited the Na\(^{+}\)-dependent Mg\(^{2+}\) extrusion with half inhibition occurring between 50 and 200 μM (Fig. 6), the range roughly compatible to reported IC\(_{50}\) values of the agent for the putative Na\(^{+}/Mg\(^{2+}\) exchange in human red blood cells (25 μM; Ref. 4), ferret red blood cells (<300 μM; Ref. 6), and rat cardiac myocytes (<80 μM; Ref. 17). Overall, the Na\(^{+}\)-dependent Mg\(^{2+}\) extrusion activity observed in the present study can be attributed to the Na\(^{+}/Mg\(^{2+}\) exchange, similar to reports in other cell types.

Conclusion. We have successfully established a mutant strain of MCT cells that can grow in the culture media containing very high Mg\(^{2+}\) concentrations (>100 mM). Optical measurements of [Mg\(^{2+}\)] c revealed enhanced Mg\(^{2+}\) extrusion activity from the 101Mg-tolerant cells that was dependent on [Na\(^{+}\)], and was inhibited by imipramine. These properties of the Mg\(^{2+}\) transport are consistent with those reported for the Na\(^{+}/Mg\(^{2+}\) exchange in various cell types. We conclude that the 101Mg-tolerant cells established in the present study may be useful to identify Mg\(^{2+}\) transporter molecules and to understand molecular mechanisms of the Na\(^{+}\)-dependent transport of Mg\(^{2+}\).

ACKNOWLEDGMENTS

The authors are indebted to Prof. J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for review of this manuscript.

GRANTS

This study was supported by grants from The Salt Science Research Foundation (0039), the Uehara Memorial Foundation, a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (14370016), and “High-Tech Research Center” Project for Private Universities.

REFERENCES

7. Günther T and Vormann J. Mg\(^2+\)/H\(^+\) efflux is accomplished by an amiloride-sensitive Na\(^+\)/H\(^+\)/Mg\(^2+\)/H\(^+\) antiport. *Biochem Biophys Res Commun* 130: 540–545, 1985.