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Characterization of Mg^{2+} efflux from rat erythrocytes non-loaded with Mg^{2+}

H. Ebel [a,*], T. Günther [b]

[a] *Institut für Klinische Physiologie, Klinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany*
[b] *Institut für Molekularbiologie und Biochemie, Klinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany*

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Abstract

Non-Mg^{2+}-loaded rat erythrocytes with a physiological level of Mg_i^{2+} exhibited Mg^{2+} efflux when incubated in nominally Mg^{2+}-free media. Two types of Mg^{2+} efflux were shown: (1) An Na^+-dependent Mg^{2+} efflux in NaCl and Na gluconate medium, which was inhibited by amiloride and quinidine, as was Na^{2+}/Mg^{2+} antiport in Mg^{2+}-loaded rat erythrocytes; and (2) an Na^+-independent Mg^{2+} efflux in sucrose medium and choline Cl medium, which may be differentiated into SITS-sensitive Mg^{2+} efflux at low Cl_o^- (in sucrose) and into SITS-insensitive Mg^{2+} efflux at high Cl_o^- (in 150 mmol/l choline Cl).

1. Introduction

Most cell types display only a low and incomplete rate of isotopic ^{28}Mg–^{24}Mg exchange [1–3] and almost no or hardly measurable net Mg^{2+} efflux when incubated in a Mg^{2+}-free medium. For example, erythrocytes from man [4] and ferret [5] exhibited a low Mg^{2+} efflux, amounting to 7 μmol/l cell/h [4] or 41 μmol/l cells/h [5], respectively. Similarly, in non-Mg^{2+}-loaded lymphocytes, no Na^+/Mg^{2+} antiport [6] or only a low rate of Na^+/Mg^{2+} antiport of about 6 nmol/mg protein/30 min [7] was found. Thus, so far, it has not been clarified which cell types express Mg^{2+} efflux without Mg^{2+} loading and which is the mechanism of Mg^{2+} efflux from non-Mg^{2+}-loaded cells.

However, after loading erythrocytes with Mg^{2+}, a high rate of Mg^{2+} efflux via Na^+/Mg^{2+} antiport could be induced (for review see [8–10]) which, due to species differences, is in the range of 1–19 mmol/l cells/h [11]. Furthermore, it was found that there was an Na^+-independent Mg^{2+} efflux. Mg^{2+} efflux via Na^+/Mg^{2+} antiport could be inhibited by amiloride and quinidine, while Na^+-independent Mg^{2+} efflux was inhibited by extracellular Cl^- and by SITS [11–14]. Whether the properties of Mg^{2+} efflux in Mg^{2+}-loaded and in non-Mg^{2+}-loaded cells are the same is an open question.

Abbreviations: ATP_i, intracellular concentration of adenosine triphosphate; Cl_o^-, extracellular concentration of Cl^-; dB-cAMP, dibutyryl-cyclo-adenosine monophosphate; Mg_i^{2+}, intracellular concentration of free Mg^{2+}; Mg_o^{2+}, extracellular concentration of Mg^{2+}; MIA, methyl-isobutyl-amiloride; Na_o^+, extracellular concentration of Na^+; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TCA, trichloro acetic acid

* Corresponding author. Fax: +49-30-8445-4239;
E-mail: ebel@ukbf.fu-berlin.de

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Comparing Mg^{2+}-loaded erythrocytes from various species, it was found that they exhibited very different rates of Mg^{2+} efflux [11,15]. In Mg^{2+}-loaded rat erythrocytes, Mg^{2+} efflux was approximately 50 times higher than in Mg^{2+}-loaded human erythrocytes [11]. The high Mg^{2+} efflux rate of Mg^{2+}-loaded rat erythrocytes encouraged us to investigate and characterize the Mg^{2+} efflux of rat erythrocytes with normal Mg^{2+} content.

2. Materials and methods

2.1. Materials

Nembutal-sodium (pentobarbital-sodium) 50 mg/ml was from Abbott, North Chicago, Il, USA, amiloride hydrochloride, dB-cAMP, SITS and furosemide were from Sigma, Deisenhofen, Germany. All other chemicals were purchased at the highest grade of purity available from Merck (Darmstadt, Germany). Filtered, de-ionized water with a resistance of 15–18 MΩ/cm, which was virtually Mg^{2+}-free, was used for solutions.

2.2. Red cell preparation

Blood (6–8 ml) was obtained from single anesthetized (50 mg/kg Nembutal-sodium i.p.) male Wistar rats (weighing 350–450 g) by catheterizing the abdominal vein with a heparinized syringe. Portions of the collected blood were transferred to heparinized tubes, diluted 1:3–1:5 with a solution of 150 mmol/l NaCl containing 5 mmol/l D-glucose and 10 mmol/l HEPES-Tris, pH 7.4. The cell suspension was centrifuged at $1000\times g$ for 10 min at 20°C. The plasma and buffy coat containing the white cells were aspirated and discarded. The red cell pellets were washed twice at 20°C in 10 ml of the buffered NaCl-glucose medium. Finally, the pellets were suspended as a 10% suspension in one of the following media, each containing 5 mmol/l D-glucose and 10 mmol/l HEPES-Tris of pH 7.4: (a) 150 mmol/l NaCl; (b) 150 mmol/l Na gluconate; (c) 150 mmol/l choline Cl; or (d) 300 mmol/l sucrose. While investigating the effect of various substances, paired experiments were always performed in the different media.

2.3. Mg^{2+} efflux

At the beginning of incubation (approximately at 1 min) and then after 10, 60 and 120 min, 1-ml aliquots of the cell suspensions were centrifuged at $1000\times g$ for 10 min. This relatively low speed was used to minimize hemolysis. For Mg^{2+} determination, 500 μl supernatant was diluted with TCA. The final concentration of TCA was 5% (w/v), containing 0.1% (w/v) La_2O_3 and 0.16% (v/v) HCl. Mg^{2+} was measured in triplicate by atomic absorption spectrometry (Perkin–Elmer, 2380). Mg^{2+} efflux was calculated from the difference in the increase of extracellular Mg^{2+} concentration during the time intervals and was related to the original cell volume measured by hematocrit. Na^+-dependent Mg^{2+} efflux was obtained by Mg^{2+} efflux in NaCl medium minus Mg^{2+} efflux in choline Cl medium. Na^+-independent Mg^{2+} efflux was defined as Mg^{2+} efflux in choline Cl medium or in sucrose medium.

Analyses were corrected for Mg^{2+} loss due to hemolysis by determining hemoglobin at 577 nm. Emphasis was laid on rather gentle handling of erythrocytes to avoid hemolysis, which ranged only between 0.6 and 1.5%. Centrifugation in the cold and above $1000\times g$, increasing the HEPES-Tris buffer of pH 7.4 above 10 mmol/l and number of pipettings increased hemolysis. Hematocrit was determined by centrifugation of blood containing capillaries at $1500\times g$ for 10 min.

2.4. Statistical analysis

Data were expressed as mean values ± S.E.M. and statistical differences were determined by Student's paired and two-tailed t-test. A value of $P<0.05$ was considered significant.

3. Results

3.1. Na^+-dependent and Na^+-independent Mg^{2+} efflux

As can be seen in Fig. 1 and Table 1 (in which all series of experiments are listed), rat erythrocytes nonloaded with Mg^{2+} showed an efflux of Mg^{2+} into nominal Mg^{2+}-free NaCl-, choline Cl- and sucrose-

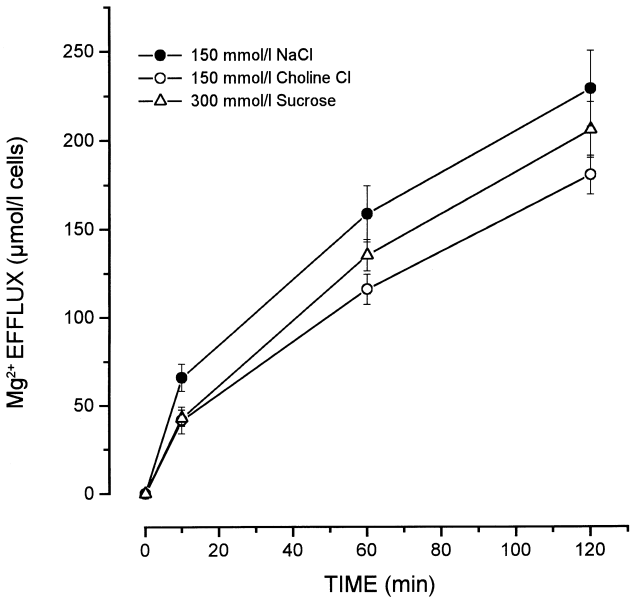

Fig. 1. Time course of Mg^{2+} efflux from non-Mg^{2+}-loaded rat erythrocytes at 37°C. A 10% (v/v) cell suspension was incubated in NaCl medium, choline Cl medium, and sucrose medium. Mean value ± S.E.M., $n = 5–7$.

medium. Thus Mg^{2+} efflux could be differentiated into: (a) Na^+-dependent Mg^{2+} efflux (Mg^{2+} efflux in NaCl medium minus choline Cl medium); and (b) Na^+-independent Mg^{2+} efflux (Mg^{2+} efflux in choline Cl medium and/or in sucrose medium). During the first hour of incubation, Na^+-independent Mg^{2+} efflux in choline Cl-medium amounted to 115.9 ± 8.6 µmol/l cells/h (Fig. 1). Na^+-dependent Mg^{2+} efflux (NaCl medium−choline Cl medium) amounted to 42.7 ± 13.1 µmol/l cells/h ($n = 7$).

In addition, Na^+-dependent Mg^{2+} efflux was determined as Mg^{2+} efflux into Na gluconate medium. Mg^{2+} efflux into Na gluconate medium was almost the same as Mg^{2+} efflux into NaCl medium (Table 1). This finding is surprising. In NaCl medium, Mg^{2+} efflux consisted of an Na^+-dependent and an Na^+-independent component, the latter found in high Cl^- medium (choline Cl). Consequently, in Na gluconate medium, there should only be the Na^+-dependent component of Mg^{2+} efflux so that Mg^{2+} efflux in Na gluconate medium should be lower than in NaCl medium, which was not the case. However, it must be considered that in Cl^--free medium, as in sucrose medium [16] or media with non-permeable anions like citrate or gluconate [17], cation permeability has been reported to be considerably enhanced [16,17]. In agreement with this effect, in our study, Mg^{2+} efflux in Na^+ gluconate medium was somewhat higher than in sucrose medium (Table 1). Therefore, Mg^{2+} efflux in Na^+ gluconate medium cannot be taken as a measure for Na^+-dependent Mg^{2+} efflux. Consequently, Na^+-dependent Mg^{2+} efflux was calculated from the difference of Mg^{2+} efflux in NaCl minus choline Cl medium.

To characterize the increase of Mg^{2+} efflux by omission of Cl_o^-, as it occurs in sucrose and Na gluconate medium, Cl^--dependence of Mg^{2+} efflux was tested by a stepwise iso-osmotic substitution of sucrose in sucrose medium by choline Cl. As shown in Fig. 2, Mg^{2+} efflux decreased with increasing choline Cl up to 30 mmol/l. Inhibition of Na^+-independent Mg^{2+} efflux by choline can be excluded for the following reasons: (1) in previous experiments with Mg^{2+}-loaded human erythrocytes, Na^+-independent Mg^{2+} efflux in sucrose medium was identically inhibited when sucrose was stepwise iso-osmotically substituted by LiCl, KCl or choline Cl [12]; and (2) in

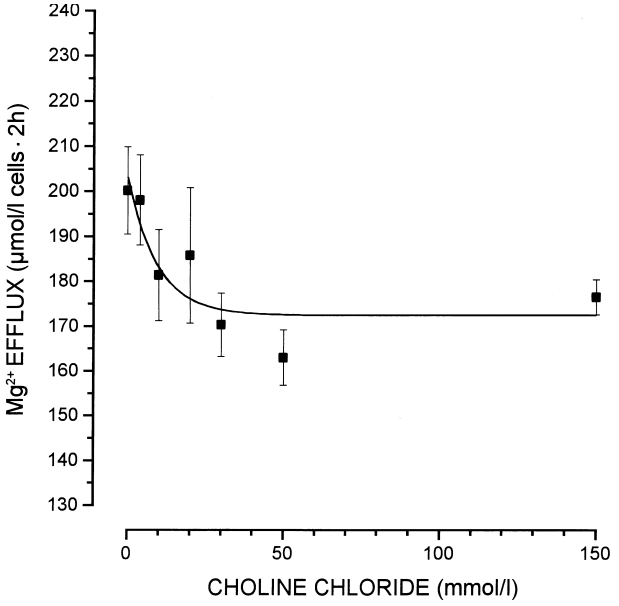

Fig. 2. Effect of extracellular choline Cl on Mg^{2+} efflux of non-Mg^{2+}-loaded rat erythrocytes. A 10% cell suspension was incubated in sucrose medium for 2 h at 37°C. The Cl^- concentration was varied by iso-osmotic substitution of sucrose by choline Cl. Mean value ± S.E.M., $n = 6–7$.

Table 1
Effect of various substances on the Mg^{2+} efflux of non Mg^{2+}-loaded rat erythrocytes

Substances	Mg^{2+} efflux (μmol/l cells/2 h)			
	NaCl	Na gluconate	Choline Cl	Sucrose
Control	209.8 ± 8.5 (n = 14)	201.7 ± 6.3 (n = 10	138.6 ± 5.4 (n = 14	189.8 ± 11.3 (n = 8)
Amiloride (0.5 mmol/l)	157.8 ± 7.8[a] (n = 9)	161.5 ± 6.1 (n = 10)	52.6 ± 3.8[a] (n = 9)	96.7 ± 3.3[a] (n = 6)
Methyl-isobutyl-amiloride (MIA; 0.1 mmol/l)	179.0 ± 5.6[a] (n = 7)	163.7 ± 12.4[a] (n = 7)	78.4 ± 7.0[a] (n = 6)	120.0 ± 13.0[a] (n = 6)
Quinidine (0.1 mmol/l)	118.6 ± 10.6[a] (n = 5)	126.3 ± 9.8[a] (n = 6)	54.3 ± 3.2[a] (n = 5)	68.4 ± 5.6[a] (n = 5)
dB-cAMP (0.5 mmol/l)	219.0 ± 14.0[a] (n = 11)	199.8 ± 4.2 (n = 6)	162.9 ± 14.0[a] (n = 9)	192.2 ± 9.2 (n = 6)
SITS (0.1 mmol/l)	200.1 ± 12.3 (n = 6)	186.2 ± 13.5 (n = 6)	141.1 ± 8.0 (n = 6)	138.7 ± 10.1[a] (n = 6)
Furosemide (0.1 mmol/l)	216.1 ± 7.0 (n = 5)	198.9 ± 8.4 (n = 6)	132.1 ± 5.9 (n = 5)	180.3 ± 7.7 (n = 5)

A 10% cell suspension was incubated for 2 h at 37°C in nominally Mg^{2+}-free NaCl, Na gluconate, choline Cl and sucrose medium. Results of paired experiments were expressed as mean value ± S.E.M. n = number of experiments.
[a] $P < 0.05$.

the present experiments with non-Mg^{2+}-loaded rat erythrocytes, 150 mmol/l KCl reduced Mg^{2+} efflux to a similar level as did 150 mmol/l choline Cl (KCl 154.5 ± 8.0 μmol/l cells/2 h, n = 6 and choline Cl 176.5 ± 3.9 μmol/l cells/2 h, n = 7 in the series of Fig. 2).

Earlier, a reduction of K^+ efflux by Cl^- was also found [16,17]. This was explained by a loss of anion selectivity of capnophorin at low Cl_o^- so that capnophorin may act as a low conductivity channel for cations [17]. Thus it can be concluded that Mg^{2+} efflux in Na gluconate medium may consist of two components: (1) Na^+-dependent Mg^{2+} efflux via Na^+/Mg^{2+} antiport; and (2) Mg^{2+} efflux induced by reduction or omission of Cl_o^-.

An important criterion for Na^+-dependent Mg^{2+} efflux via Na^+/Mg^{2+} antiport is the stoichiometric ratio of Na^+:Mg^{2+}. However, because of the low rate of Mg^{2+} efflux and consequently of the small increase in intracellular Na^+ (see Section 4), the stoichiometric ratio of Na^+ uptake/Mg^{2+} efflux has not been determined.

3.2. Effect of amiloride

Next we investigated whether the properties of the Na^+-dependent and Na^+-independent components of Mg^{2+} efflux from non-Mg^{2+}-loaded cells were the same as those of Mg^{2+}-loaded erythrocytes. In previous studies with Mg^{2+}-loaded human and rat erythrocytes, both the Na^+-dependent [4,9,14,18,19] as well as the Na^+-independent component [12,13,19] of Mg^{2+} efflux could be inhibited by amiloride. Fig. 3 and Table 1 show the effect of 0.5 mmol/l amiloride on non-Mg^{2+}-loaded rat erythrocytes. At this concentration, amiloride inhibited Mg^{2+} efflux in all media by 20–62%. At 0.1 mmol/l methyl-isobutyl-amiloride (MIA) a more potent inhibitor of Na^+ channels, Na^+/H^+-, Na^+/Ca^{2+}- and of Na^+/Mg^{2+}-exchange [20–23] also inhibited Mg^{2+} efflux in these media. Amiloride inhibition of Mg^{2+} efflux in Na gluconate medium was smaller than in the other media. This agrees with the previous finding of a different inhibition of Na^+/H^+ antiport by amiloride in NaCl and Na gluconate medium [24]. Thus amiloride

showed the same effects on Na^+-dependent and Na^+-independent Mg^{2+} efflux in non-Mg^{2+}-loaded as in Mg^{2+}-loaded erythrocytes [11,12].

3.3. Effect of quinidine

In previous studies with Mg^{2+}-loaded human erythrocytes, it has been shown that only Na^+-dependent Mg^{2+} efflux can be inhibited by quinidine [5,11], while in Mg^{2+}-loaded rat erythrocytes both components of Mg^{2+} efflux were sensitive to inhibition by quinidine [11]. In Fig. 4 and Table 1, the effect of 0.5 mmol/l quinidine on Mg^{2+} efflux of non-Mg^{2+}-loaded erythrocytes can be seen. As was the case with amiloride, quinidine too inhibited both components of Mg^{2+}efflux of non-Mg^{2+}-loaded rat erythrocytes, as was found with Mg^{2+}-loaded rat erythrocytes [11].

3.4. Effect of dB-cAMP

While in Ehrlich ascites tumor cells [7], lymphocytes [25], hepatocytes [26] and ventricular myocytes [27] cAMP could increase Na^+-dependent Mg^{2+} efflux, it was reported to have no effect on Mg^{2+} efflux of non-Mg^{2+}-loaded human erythrocytes in NaCl medium [28] or on reversed Na^+/Mg^{2+} antiport of Mg^{2+}-loaded rat erythrocytes [29]. In the present study with non-Mg^{2+}-loaded rat erythrocytes, 0.5 mmol/l dB-cAMP stimulated Mg^{2+} efflux in NaCl medium by 4.4% and in choline Cl medium by 17.5%, while in Na gluconate and sucrose medium no significant effect of dB-cAMP was found (Table 1). The effect of dB-cAMP on Na^+-independent Mg^{2+} efflux needs further consideration.

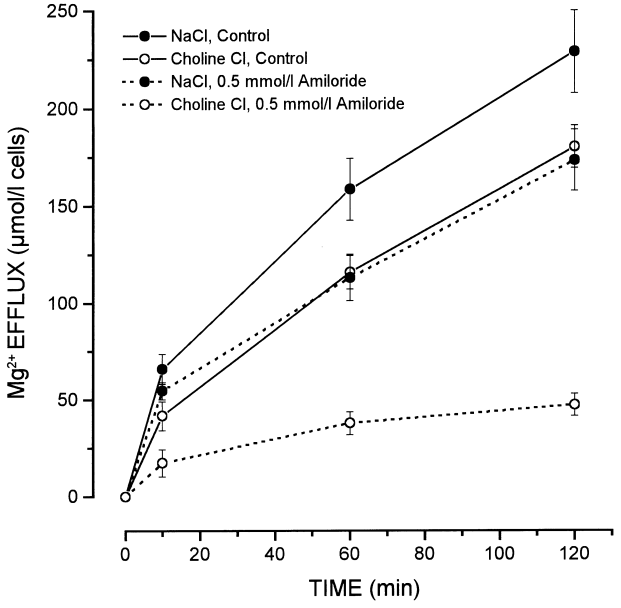

Fig. 3. Effect of amiloride (0.5 mmol/l) on Mg^{2+} efflux of non-Mg^{2+}-loaded rat erythrocytes in NaCl medium and choline Cl medium. Mean value ± S.E.M., $n = 5–7$. Values in the presence of amiloride at all times differ significantly from control values ($P < 0.05$).

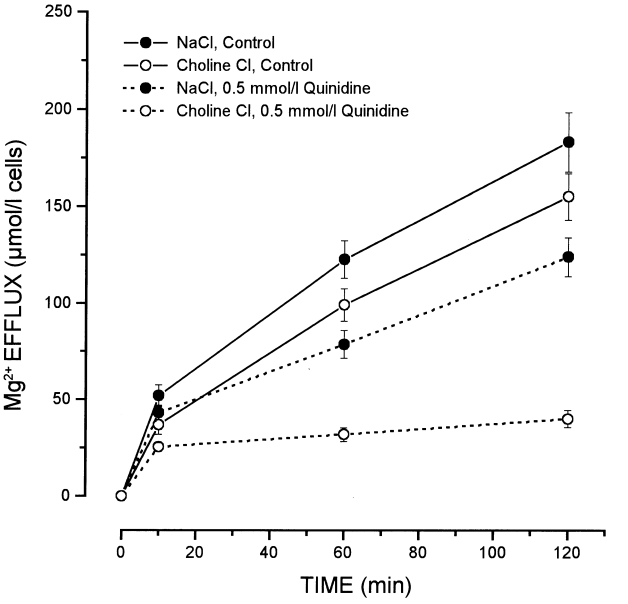

Fig. 4. Effect of quinidine (0.1 mmol/l) on Mg^{2+} efflux of non-Mg^{2+}-loaded rat erythrocytes in NaCl medium and choline Cl medium. Mean value ± S.E.M., $n = 5–7$. Values in the presence of quinidine are at all times significantly different from control values ($P < 0.05$).

3.5. Effect of SITS

Earlier it has been found that Na^+-independent Mg^{2+} efflux of Mg^{2+}-loaded rat, human and chicken erythrocytes was inhibited by stilbene derivates as SITS and DIDS, but only at low Cl_o^- [11–13]. In this study, with non-Mg^{2+}-loaded rat erythrocytes, 0.1 mmol/l SITS had no effect on Mg^{2+} efflux in high Cl^- medium (NaCl, choline Cl) or Na gluconate medium (see Table 1). Only in sucrose medium was Mg^{2+} efflux inhibited by SITS, which is similar to preceding results with Mg^{2+}-loaded rat erythrocytes [11].

3.6. Effect of furosemide

Mg^{2+} efflux from Mg^{2+}-loaded human erythrocytes was found to be insensitive to furosemide and bumetanide [4,12], which both inhibit the Na,K,Cl symporter or the K,Cl symporter. In this study with rat erythrocytes, non-loaded with Mg^{2+}, 0.1 mmol/l furosemide did not inhibit Mg^{2+} efflux in any of the used media (Table 1) as was the case in Mg^{2+}-loaded human erythrocytes [4,12].

4. Discussion

Our present experiments with non-Mg^{2+}-loaded rat erythrocytes have shown a significant Na^+-dependent and Na^+-independent Mg^{2+} efflux when Mg^{2+} efflux was measured in nominally Mg^{2+}-free NaCl and Na gluconate medium or in Mg^{2+}-free choline Cl and sucrose medium. In previous experiments, similar results were found with Mg^{2+}-loaded rat erythrocytes [8–13]. Also, the effect of various effectors on both components of Mg^{2+} efflux was similar in non-Mg^{2+}-loaded and Mg^{2+}-loaded rat erythrocytes [8–13]. Therefore, the mechanism of Na^+-dependent and Na^+-independent Mg^{2+} efflux in Mg^{2+}-loaded and non-Mg^{2+}-loaded rat erythrocytes may be similar.

In Mg^{2+}-loaded cells, Na^+-independent Mg^{2+} efflux in sucrose or low Cl^- medium was explained as Mg^{2+} transport driven by the intra/extracellular Mg^{2+} gradient or electrochemical potential and accompanied by efflux of Cl^- for charge compensation, which can be inhibited by high extracellular Cl^- and by DIDS or SITS [12,13]. The finding of this study, that in non-Mg^{2+}-loaded rat erythrocytes the rate of Na^+-independent Mg^{2+} efflux in choline Cl^- medium (Fig. 1) was 9.5 times lower at 30 min than in rat erythrocytes loaded with Mg^{2+} in the presence of 12 mmol/l Mg^{2+} (Table 2 of [11]), is in agreement with this mechanism. At least in human erythrocytes it has been shown that after Mg^{2+} loading by means of A 23187, Mg_i^{2+} can be increased in parallel to Mg_o^{2+} [30].

The increase in Mg^{2+} efflux when choline Cl was substituted by sucrose (Fig. 2), may be due to an increase in the intracellular/extracellular Cl^- gradient and thus an increased Cl^- efflux which accompanies Mg^{2+} efflux for charge compensation. Mg^{2+} efflux in sucrose medium was inhibited by SITS. It is therefore possible that the Cl^- efflux accompanying the Mg^{2+} efflux is operating via capnophorin. Alternatively, it may be argued that in low Cl_o^- media or Cl_o^--free media, Mg^{2+} may permeate through capnophorin as suggested for K^+ efflux in low Cl_o^- media [16].

Na^+-independent Mg^{2+} efflux in 150 mmol/l choline Cl medium was not inhibited by SITS (Table 1). This result may indicate the existence of a second component of Na^+-independent Mg^{2+} efflux. In human erythrocytes, an unspecific transporter has been described that exchanges choline for other cations [31]. Whether in rat erythrocytes the SITS-insensitive Mg^{2+} efflux in choline Cl medium may operate via this unspecific choline exchanger and whether this transporter may be inhibited by amiloride and quinidine, requires further study.

The interpretation of Mg^{2+} efflux in gluconate and sucrose medium is complicated since under these conditions Cl^- leaves the cell, the membrane potential is reversed and intracellular pH increases. Also Na^+ and K^+ fluxes increase [16,17,32,33]. Further experiments are needed to clarify these mechanisms.

Na^+-dependent Mg^{2+} efflux in Mg^{2+}-loaded rat erythrocytes has been characterized as Na^+/Mg^{2+} antiport with a stoichiometric ratio of 2 Na^+/1 Mg^{2+} inhibited by amiloride and quinidine [8–10]. In this study with non-Mg^{2+}-loaded rat erythrocytes, the stoichiometric ratio has not been investigated because of the low transport rate. If the stoichiometric ratio is 2 Na^+/1 Mg^{2+}, the increase in intracellular Na^+ (at Na_i^+ of about 10 mmol/l) should amount to 0.1 mol/l cells/h, which is hardly measurable.

When we compare the Na^+-dependent Mg^{2+} efflux of Mg^{2+}-loaded rat erythrocytes, loaded with Mg^{2+} at $Mg_o^{2+} = 12$ mmol/l (8.9 mmol/l cells/30 min from Table 2 of [11]), with Na^+-dependent Mg^{2+} efflux from non-Mg^{2+} loaded rat erythrocytes of this study (0.021 mmol/l cells/30 min from Fig. 1), the ratio of Mg^{2+} efflux of Mg^{2+} loaded/non-Mg^{2+} loaded erythrocytes was about 400. The analogous ratio for Na^+-independent Mg^{2+} efflux was only about 9.5 (see above). A similar drastic increase of Na^+-dependent Mg^{2+} efflux was found with ferret erythrocytes after loading the cells with Mg^{2+}. In ferret erythrocytes,

there was low or almost no Mg^{2+} efflux when Mg_i^{2+} was below 0.9 mmol/l, while above this threshold Mg^{2+} efflux was dramatically increased [34].

With respect to the very similar or identical actions of various effectors, there should be a close relationship between the Na^+/Mg^{2+} antiport in Mg^{2+}-loaded rat erythrocytes and that of the Na^+-dependent Mg^{2+} efflux in non-Mg^{2+}-loaded rat erythrocytes.

The following model has been suggested for Mg^{2+} transport [35]: in non-Mg^{2+} loaded cells, the Mg^{2+} transporter should only perform Mg^{2+}/Mg^{2+} exchange or Mg^{2+}/Mn^{2+} exchange but no net Mg^{2+} efflux. Mg^{2+} loading would be associated with the binding of 2 Mg^{2+} ions to an intracellular regulatory site of the Mg^{2+} transporter, which may be favored by ATP_i. This would induce an allosteric transition of the Mg^{2+} transporter, which then could perform exchange of intracellular Mg^{2+} for extracellular Na^+.

As the present study clearly showed an Na^+-dependent Mg^{2+} efflux in non-Mg^{2+}-loaded rat erythrocytes, we now suggest that the Mg^{2+} transporter can perform a low rate of Na^+/Mg^{2+} exchange at physiological levels of Mg_i^{2+}. Due to its low activity and/or higher specificity, Na^+/Mg^{2+} exchange may not be measurable in several cell types. Nevertheless, in all cell types, after Mg^{2+} loading, the Mg^{2+} transporter may be allosterically activated to perform a high rate of Na^+/Mg^{2+} antiport. Alternatively, it may be argued that, after Mg^{2+} loading, an activator protein is bound to the Mg^{2+} transport protein thus increasing its activity. Binding of an activator protein may depend on Mg_i^{2+} and ATP_i. A similar mechanism has been discussed for the Na^+/H^+ antiporter [36,37].

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