

# Na<sup>+</sup>-independent Mg<sup>2+</sup> efflux from Mg<sup>2+</sup>-loaded human erythrocytes

T. Günther and J. Vormann

*Institute of Molecular Biology and Biochemistry, Free University of Berlin, Arnimallee 22, D-1000 Berlin 33, Germany*

Received 16 February 1989

Net Mg<sup>2+</sup> efflux from Mg<sup>2+</sup>-loaded human erythrocytes was maximal after reincubation in sucrose. Net Mg<sup>2+</sup> efflux was not inhibited by furosemide or bumetanide and, therefore, was not performed by the (Na,K,Cl)- or (K,Cl)-cotransport system. A component of net Mg<sup>2+</sup> efflux was inhibited by extracellular NaCl, KCl, LiCl, choline Cl and SITS, in analogy to the inhibition of net Cl<sup>-</sup> and SITS. Therefore, it was concluded that net Mg<sup>2+</sup> efflux is dependent on net Cl<sup>-</sup> efflux for charge compensation. Cl<sup>-</sup>-dependent net Mg<sup>2+</sup> efflux was inhibited by amiloride. Only 10% of the maximal net Mg<sup>2+</sup> efflux may depend on extracellular Na<sup>+</sup>.

Mg<sup>2+</sup> efflux; Na<sup>+</sup>; Cl<sup>-</sup>; Acetamido-4'-isothiocyanatostilbene-2, 2'-disulfonic acid, 4-; Amiloride; (Human erythrocyte)

## 1. INTRODUCTION

With Mg<sup>2+</sup>-loaded chicken erythrocytes we found an Mg<sup>2+</sup> efflux which was coupled to Na<sup>+</sup> influx and was inhibited by high doses of amiloride [1–3]. The results were explained by the action of Na<sup>+</sup>/Mg<sup>2+</sup> antiport.

The experiments were repeated with human [4–6] and rat [7] erythrocytes, and it was reported that Mg<sup>2+</sup> efflux from human erythrocytes was also dependent on extracellular Na<sup>+</sup> [4–6] and was inhibited by amiloride [5]. However, there were controversial conclusions on the role of Na<sup>+</sup>. Féray and Garay [6] found that in human erythrocytes 3 Na<sup>+</sup> were exchanged for 1 Mg<sup>2+</sup>, whereas Lüdi and Schatzmann [5] were reluctant to explain Na<sup>+</sup> dependency of net Mg<sup>2+</sup> efflux

from human erythrocytes by simple Na<sup>+</sup>/Mg<sup>2+</sup> exchange.

Therefore, we investigated in greater detail the role of Na<sup>+</sup> in Mg<sup>2+</sup> efflux from Mg<sup>2+</sup>-loaded human erythrocytes. However, our results showed the existence of Na<sup>+</sup>-independent net Mg<sup>2+</sup> efflux from human erythrocytes, accompanied by net Cl<sup>-</sup> efflux for charge compensation.

## 2. MATERIALS AND METHODS

All experiments were performed with human erythrocytes from one donor (J.V.). Blood was taken with a heparinized syringe by venous puncture and centrifuged at 1000 × g for 10 min. The plasma and buffy coat were aspirated and the red cells were washed twice with 150 mM KCl.

The cells were loaded with Mg<sup>2+</sup> by incubating a 10% cell suspension for 30 min at 37°C in KCl medium (140 mM KCl, 12 mM MgCl<sub>2</sub>, 50 mM sucrose, 5 mM glucose, 30 mM Hepes/Tris, pH 7.4) with the addition of 6 μM A23187 dissolved in dimethyl sulfoxide. For removal of the ionophore the cells were incubated four times in KCl medium plus 1% bovine serum albumin for 10 min at 37°C. The KCl medium was removed by washing the cells twice with cold (4°C) sucrose medium (350 mM sucrose, 5 mM glucose, 30 mM Hepes/Tris, pH 7.4).

Mg<sup>2+</sup> and K<sup>+</sup> efflux was measured by reincubating a 10% cell suspension at 37°C in Mg<sup>2+</sup>-free medium. For reincubation sucrose medium, unbuffered 350 mM sucrose or XCl media

*Correspondence address:* T. Günther, Institute of Molecular Biology and Biochemistry, Free University of Berlin, Arnimallee 22, D-1000 Berlin 33, Germany

*Abbreviations:* Na<sup>+</sup>, Cl<sup>-</sup>, extracellular concentrations of Na<sup>+</sup> or Cl<sup>-</sup>; TCA, trichloroacetic acid; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid

(substitution of KCl medium by XCl) were used, as indicated. At the beginning of reincubation and after 30 min, 0.5 ml aliquots of the cell suspension were centrifuged for 1 min at  $10000 \times g$ .

For  $Mg^{2+}$  determination, 100  $\mu$ l supernatant was diluted with 1 ml of 10% TCA/0.175%  $LaCl_3$  and  $Mg^{2+}$  was measured by atomic absorption spectrophotometry (Philips, SP9).  $K^+$  content in the supernatant was measured after addition of appropriate amounts of LiCl by flame photometry (KLiNa-Flame, Beckman). An aliquot of the supernatant was taken for determination of hemoglobin by means of the cyanohemoglobin method [1].

For measuring cellular  $Mg^{2+}$  content, cells were washed twice with 150 mM KCl and hemolysed by adding 750  $\mu$ l  $H_2O$ . 50  $\mu$ l of the hemolysate were taken for determination of hemoglobin, the rest was deproteinized by addition of 50  $\mu$ l of 75% TCA, centrifuged and the  $Mg^{2+}$  content was measured by atomic absorption spectrophotometry after dilution with 10% TCA/0.175%  $LaCl_3$ . Cellular  $Mg^{2+}$  content was taken to correct  $Mg^{2+}$  efflux for hemolysis.

### 3. RESULTS AND DISCUSSION

Contrarily to chicken erythrocytes, in which only a small part of  $Mg^{2+}$  efflux was independent of  $Na^+$  [2], in  $Mg^{2+}$ -loaded human erythrocytes  $Mg^{2+}$  efflux was highest in sucrose (fig.1). Also contrarily to chicken erythrocytes, in human erythrocytes,  $Mg^{2+}$  efflux was inhibited by NaCl (fig.1).

The inhibition of  $Mg^{2+}$  efflux was not specific for NaCl. KCl and LiCl also inhibited  $Mg^{2+}$  efflux when sucrose was isoosmotically substituted by these salts (fig.1). Inhibition by alkali chlorides increased up to 50 mM and remained nearly constant up to 150 mM. In 150 mM NaCl  $Mg^{2+}$  efflux was inhibited by 54%, whereas in 150 mM KCl or LiCl,  $Mg^{2+}$  efflux was inhibited by 64% (fig.1).

As shown by the Dixon plot,  $Mg^{2+}$  efflux was noncompetitively inhibited by extracellular KCl and LiCl,  $K_i$  amounted to 24 mM (fig.2).

$Mg^{2+}$  efflux from human erythrocytes was independent of the osmolarity of the medium (fig.3).

Since  $Mg^{2+}$  efflux was highest in sucrose medium or in the absence of extracellular ions, we looked for ions accompanying net  $Mg^{2+}$  efflux. Under the conditions of net  $Mg^{2+}$  efflux from  $Mg^{2+}$ -loaded human erythrocytes, we found net efflux of  $K^+$ . In sucrose medium the molar ratio of  $K^+$  efflux: $Mg^{2+}$  efflux amounted to 30:1, and in NaCl medium this ratio amounted to 4:1.

The high rate of net  $Mg^{2+}$  and  $K^+$  efflux must be accompanied by anions for charge compensation.

$K^+$  can leave the cell by (Na,K,Cl)- or by (K,Cl)-cotransport [8].

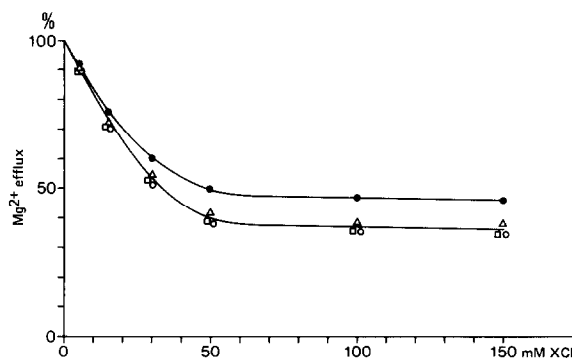


Fig.1. Inhibition of net  $Mg^{2+}$  efflux from human erythrocytes by chlorides. Sucrose was isoosmotically substituted by NaCl (●), LiCl (Δ), KCl (○), and choline Cl (□). 100% value of  $Mg^{2+}$  efflux was measured in sucrose medium and amounted to  $0.99 \pm 0.20$  mmol/l cells per 30 min. Mean  $\pm$  SE of 4 experiments.

To prove whether  $Mg^{2+}$  may be transported out of human erythrocytes by these cotransport systems, we tested the effect of furosemide and bumetanide on  $Mg^{2+}$  efflux. As shown in table 1, both inhibitors had no significant effect on  $Mg^{2+}$  efflux either in sucrose or in NaCl medium. Therefore, it can be concluded that  $Mg^{2+}$  is not transported out of human erythrocytes by these cotransport systems. Another transport system must perform net  $Mg^{2+}$  efflux in these cells.

For charge compensation net  $Mg^{2+}$  and  $K^+$  efflux should be accompanied by net efflux of  $Cl^-$ . When  $Mg^{2+}$  is leaving the cell with  $Cl^-$ , the inhibition of net  $Mg^{2+}$  efflux by NaCl, KCl and LiCl may represent the primary inhibition of net  $Cl^-$  efflux which is inhibited by extracellular  $Cl^-$  [9,10]. To test this effect of  $Cl^-$ , we measured the influence of choline Cl on net  $Mg^{2+}$  efflux. As also shown in fig.1, choline Cl inhibited net  $Mg^{2+}$  efflux in the same way as KCl and LiCl, indicating that  $Cl^-$  is the inhibitor of net  $Mg^{2+}$  efflux.

The mechanism by which  $Cl^-$  inhibits net  $Mg^{2+}$  efflux may be the same as the inhibition of net  $K^+$  efflux and KCl efflux from human red cells by  $Cl^-$ . Net KCl or  $Cl^-$  efflux from human erythrocytes was reduced when  $Cl^-$  was increased to 50 mM and was essentially constant between 50 and 150 mM  $Cl^-$  [10].

As shown in fig.1,  $Cl^-$  dependency of net  $Mg^{2+}$  efflux behaved identically to the  $Cl^-$  dependency of net  $Cl^-$  efflux.

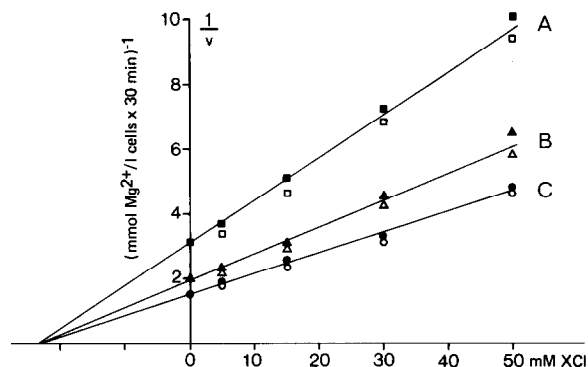


Fig.2. Dixon plot of net  $\text{Mg}^{2+}$  efflux from human erythrocytes in KCl ( $\bullet$ ,  $\blacktriangle$ ,  $\blacksquare$ ) and LiCl ( $\circ$ ,  $\triangle$ ,  $\square$ ) medium. The cells were loaded with  $\text{Mg}^{2+}$  at extracellular  $\text{Mg}^{2+}$  concentrations of 4 (A), 8 (B) and 12 (C) mM. The corresponding cellular  $\text{Mg}^{2+}$  contents amounted to 7.0 (A), 12.2 (B), and 16.0 (C) mmol/l cells. Mean of 2 experiments.

Moreover, net  $\text{Cl}^-$  efflux from human erythrocytes can be inhibited by stilbene disulfonates [8–10]. Therefore, as further proof for the role of net  $\text{Cl}^-$  efflux, we tested the effect of SITS on net  $\text{Mg}^{2+}$  efflux. In sucrose medium, SITS inhibited net  $\text{Mg}^{2+}$  efflux by 53% (table 2).

This result shows that in sucrose there is a SITS-sensitive and a SITS-insensitive net  $\text{Mg}^{2+}$  efflux. Also, net  $\text{Cl}^-$  efflux from human erythrocytes consists of a DIDS-sensitive and DIDS-insensitive component [9,10]. Therefore, it can be concluded that SITS-sensitive net  $\text{Mg}^{2+}$  efflux in sucrose is determined by SITS-sensitive net  $\text{Cl}^-$  efflux. SITS-insensitive net  $\text{Mg}^{2+}$  efflux in sucrose may depend on SITS-insensitive net  $\text{Cl}^-$  efflux. This mechanism has not been defined so far, whereas DIDS-sensitive net  $\text{Cl}^-$  efflux from human erythrocytes is performed by band 3 protein [8].

In 150 mM NaCl, KCl, LiCl and choline Cl, 30  $\mu\text{M}$  SITS inhibited net  $\text{Mg}^{2+}$  efflux only by 5% (table 2). This result shows that at high  $\text{Cl}_o^-$ , net  $\text{Mg}^{2+}$  efflux is practically independent of SITS-sensitive net  $\text{Cl}^-$  efflux.

However, the transport mechanism by which net  $\text{Mg}^{2+}$  efflux is operating may be the same in sucrose- and  $\text{Cl}^-$ -containing media because net  $\text{Mg}^{2+}$  efflux under both conditions was inhibited by amiloride (table 1).

$\text{Cl}^-$ -dependent net  $\text{Mg}^{2+}$  efflux from human erythrocytes was independent of metabolic energy.

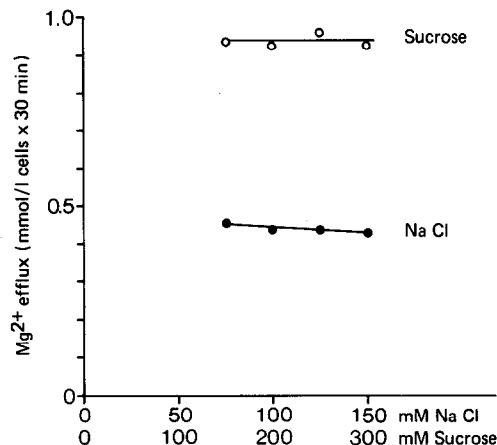


Fig.3. Net  $\text{Mg}^{2+}$  efflux from human erythrocytes reincubated in NaCl and sucrose medium with different NaCl and sucrose concentrations as indicated. Mean of 2 experiments.

Net  $\text{Mg}^{2+}$  efflux in sucrose, KCl, LiCl or choline Cl was the same when glucose was omitted from the incubation medium or when 2-deoxyglucose was added instead of glucose (data not shown). In agreement with this result, also net efflux of KCl or  $\text{Cl}^-$  from human erythrocytes was performed without addition of glucose [9,10].

$\text{Na}_o^+$  plays a minor role in net  $\text{Mg}^{2+}$  efflux from human erythrocytes. From fig.1 and table 2 it can be seen that NaCl had a smaller inhibitory effect than other chlorides.

This effect may be caused by a small rate of  $\text{Na}_o^+$ -dependent net  $\text{Mg}^{2+}$  efflux, operating simultaneously with  $\text{Cl}^-$ -dependent  $\text{Mg}^{2+}$  efflux.

Table 1

Effect of various inhibitors on net  $\text{Mg}^{2+}$  efflux from  $\text{Mg}^{2+}$ -loaded human erythrocytes incubated in sucrose medium or NaCl medium

Addition	mM	Sucrose (%)	NaCl (%)
Control	—	100	100
Furosemide	1.0	108	105
Bumetanide	0.1	100	94
Amiloride	1.0	32	44

100% value of  $\text{Mg}^{2+}$  efflux in sucrose medium amounted to  $0.93 \pm 0.17$  ( $\pm$  SE) mmol/l cells per 30 min and 100% value of  $\text{Mg}^{2+}$  efflux in NaCl medium amounted to  $0.43 \pm 0.09$  ( $\pm$  SE) mmol/l cells per 30 min. Mean of 4 experiments in duplicates

Table 2

Inhibition of net  $Mg^{2+}$  efflux from  $Mg^{2+}$ -loaded human erythrocytes in various media by 30  $\mu M$  SITS

Medium	– SITS (mmol/l cells per 30 min)	+ SITS (mmol/l cells per 30 min)
Sucrose	$0.98 \pm 0.12^a$	$0.46 \pm 0.06$
NaCl	$0.45 \pm 0.07$	$0.43 \pm 0.05$
KCl	$0.40 \pm 0.07$	$0.39 \pm 0.05$
LiCl	$0.43 \pm 0.06$	$0.41 \pm 0.06$
Choline Cl	$0.40 \pm 0.06$	$0.38 \pm 0.06$

<sup>a</sup> Mean  $\pm$  SE of 4 experiments in duplicates

When the difference between net  $Mg^{2+}$  efflux at 150 mM NaCl and 150 mM LiCl, KCl and choline Cl (fig.1) is caused by  $Na_o^+$ -dependent  $Mg^{2+}$  efflux, its rate can be calculated to be 0.2 mmol/l cells per 60 min. The same range for  $Na_o^+$ -dependent  $Mg^{2+}$  efflux was reported by Féray and Garay [4] and by Lüdi and Schatzmann [5]. These authors measured  $Na_o^+$ -dependent  $Mg^{2+}$  efflux from  $Mg^{2+}$ -loaded erythrocytes by substituting KCl for NaCl [4] or as the difference of  $Mg^{2+}$  efflux between 150 mM

NaCl and 150 mM KCl in the medium [5]. Thus,  $Na_o^+$ -dependent  $Mg^{2+}$  efflux can represent only a small fraction (10% under the condition of fig.1) of total net  $Mg^{2+}$  efflux from human erythrocytes.

## REFERENCES

- [1] Günther, T., Vormann, J. and Förster, R. (1984) *Biochem. Biophys. Res. Commun.* 119, 124–131.
- [2] Günther, T. and Vormann, J. (1985) *Biochem. Biophys. Res. Commun.* 130, 540–545.
- [3] Günther, T. and Vormann, J. (1987) *Biochem. Biophys. Res. Commun.* 148, 1069–1074.
- [4] Féray, J.C. and Garay, R. (1986) *Biochim. Biophys. Acta* 856, 75–84.
- [5] Lüdi, H. and Schatzmann, H.J. (1987) *J. Physiol.* 390, 367–382.
- [6] Féray, J.C. and Garay, R. (1988) *Naunyn-Schmiedeberg's Arch. Pharmacol.* 338, 332–337.
- [7] Féray, J.C. and Garay, R. (1987) *J. Biol. Chem.* 262, 5763–5768.
- [8] Hoffmann, E.K. (1986) *Biochim. Biophys. Acta* 864, 1–31.
- [9] Knauff, P.A., Law, F.-Y. and Marchant, P.J. (1983) *J. Gen. Physiol.* 81, 95–126.
- [10] Fröhlich, O., Leibson, C. and Gunn, R.B. (1983) *J. Gen. Physiol.* 81, 127–152.