Characterization of furosemide-sensitive Mg\textsuperscript{2+} influx in Yoshida ascites tumor cells

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Received 29 November 1985; revised version received 13 January 1986

Partially Mg\textsuperscript{2+}-depleted Yoshida ascites tumor cells took up Mg\textsuperscript{2+} after reincubation in Mg\textsuperscript{2+}- and HCO\textsubscript{3}-containing media. Mg\textsuperscript{2+} influx was insensitive to ouabain, amiloride and disulfonic stilbenes, but was non-competitively inhibited by furosemide (K\textsubscript{i} = 0.4 mM) and bumetanide. Mg\textsuperscript{2+} influx obeyed Michaelis-Menten kinetics with respect to Mg\textsuperscript{2+} concentration (K\textsubscript{m} = 1.1 mM) and was sigmoidal with respect to HCO\textsubscript{3} concentration. Electroneutral Mg\textsuperscript{2+}, HCO\textsubscript{3} cotransport was supposed to be the mechanism of Mg\textsuperscript{2+} influx.

\[ \text{Mg}^{2+} \text{ influx Furosemide sensitivity (Yoshida ascites tumor cell)} \]

1. INTRODUCTION

In [1], we described that thymocytes and ascites tumor cells, partially depleted of Mg\textsuperscript{2+}, take up Mg\textsuperscript{2+} after reincubation in Mg\textsuperscript{2+}-containing media depending on the presence of HCO\textsubscript{3}. Here, we characterize the Mg\textsuperscript{2+} uptake mechanism by means of various transport inhibitors.

2. MATERIALS AND METHODS

Yoshida ascites tumor cells were grown for 72 h at 37°C in sterile RPMI 1640 medium with addition of 10% fetal calf serum (Seromed, Munich), 25 mM Hepes, pH 7.4, 15 mM NaHCO\textsubscript{3}, 0.1 g/l streptomycin and 0.06 g/l penicillin.

For Mg\textsuperscript{2+} depletion, the ascites tumor cells (cell concentration 1%) were incubated at 37°C for 1.5 h in Na\textsuperscript{+} medium with 5 mM 2-DOG instead of 5 mM glucose. 2-DOG was phosphorylated by ATP resulting in ATP depletion and Mg\textsuperscript{2+} efflux [1]. The Na\textsuperscript{+} medium contained (in mM): 145 NaCl, 5 KCl, 1 Na\textsubscript{2}HPO\textsubscript{4}, 0.5 MgCl\textsubscript{2}, 1.2 CaCl\textsubscript{2}, 30 Hepes, pH 7.4, 5 glucose. For removal of 2-DOG the cells were centrifuged at 1000 \times g for 10 min and reincubated, as indicated in the figure legends. After removal of 2-DOG and reincubation with glucose the cellular energy state was rapidly normalized [1].

When HCO\textsubscript{3}-containing medium was used for reincubation, the medium was gassed with air-5% CO\textsubscript{2}. Some experiments were done with K\textsuperscript{+} medium, containing (in mM): 120 KCl, 20 KHCO\textsubscript{3}, 1 K\textsubscript{2}HPO\textsubscript{4}, 0.5 MgCl\textsubscript{2}, 1.2 CaCl\textsubscript{2}, 30 Hepes, pH 7.4, 5 glucose and with Cl\textsuperscript{-}-free medium, containing (in mM): 50 NaHCO\textsubscript{3}, 0.5 Mg acetate, 1 Na\textsubscript{2}HPO\textsubscript{4}, 5 glucose and 150 sucrose. The viability of the cells during Mg\textsuperscript{2+} depletion and reincubation was determined by trypan blue staining.

At various times during incubation with 2-DOG or reincubation aliquots were taken and washed with cold 0.15 M NaCl. The sedimented cells were extracted with 5% trichloroacetic acid. Mg\textsuperscript{2+} was
measured in the trichloroacetic acid extracts by atomic absorption spectrophotometry and related to protein content, measured according to Lowry et al. [2]. The protein content of the cells amounted to 0.12 g/g wet wt.

3. RESULTS AND DISCUSSION

Mg$^{2+}$ efflux is inhibited by amiloride [3]. We therefore investigated the effect of amiloride on Mg$^{2+}$ uptake. As shown in fig.1, amiloride did not inhibit Mg$^{2+}$ uptake, indicating that Mg$^{2+}$ influx operates by a mechanism other than Mg$^{2+}$ efflux.

However, Mg$^{2+}$ influx was inhibited by furosemide. Furosemide inhibition of Mg$^{2+}$ influx was noncompetitive with respect to [Mg$^{2+}$]o (fig.2, $K_i = 0.4$ mM). Fig.2 also shows that Mg$^{2+}$ uptake obeys Michaelis-Menten kinetics, with a $K_m$ for Mg$^{2+}$ amounting to 1.1 mM.

Furosemide and bumetanide inhibit Na$^+$,K$^+$,Cl$^-$ cotransport and Na$^+$,HCO$_3^-$/Cl$^-$ exchange, the latter operating via the anion-exchange system, whereas both transport systems are not inhibited by amiloride and ouabain [4–7].

As shown in fig.1, Mg$^{2+}$ uptake was insensitive to amiloride and ouabain, but was inhibited by furosemide and bumetanide. Thus, Mg$^{2+}$ uptake was affected by these inhibitors similar to the

![Fig.1. Re-uptake of Mg$^{2+}$ in Mg$^{2+}$-depleted Yoshida ascites tumor cells. Cells were reincubated in Na$^+$ medium with: 20 mM HCO$_3^-$ (○), 20 mM HCO$_3^-$ and 0.1 mM ouabain (●), 20 mM HCO$_3^-$ and 1 mM amiloride (△), 20 mM HCO$_3^-$ and 1 mM furosemide (△), 20 mM HCO$_3^-$ and 1 mM bumetanide (Δ). Mean of 3 experiments.](image)

![Fig.2. Double-reciprocal plot of Mg$^{2+}$ influx (1/v) vs 1/[Mg$^{2+}$]o. Mg$^{2+}$-depleted Yoshida ascites tumor cells were reincubated in Na$^+$ medium with 20 mM HCO$_3^-$ and different Mg$^{2+}$ concentrations. Incubation without furosemide (○), in the presence of 0.5 mM furosemide (●).](image)

![Fig.3. Rate of intracellular Mg$^{2+}$ uptake in Mg$^{2+}$-depleted Yoshida ascites tumor cells. Cells were incubated in Na$^+$ medium with different HCO$_3^-$ concentrations. Incubation without furosemide (○), in the presence of 0.3 mM furosemide (●). Mean of 3 experiments. The pH of the medium with 2.5, 5, 15, and 30 mM HCO$_3^-$ amounted to 7.40; pH in the medium with 50 mM HCO$_3^-$ amounted to 7.46.](image)
Mg$^2+$ influx, a straight line with a slope of $n = 2$ was obtained (fig.4). A Hill coefficient ($n$) of 2 indicates that 2 HCO$_3^-$ are simultaneously needed in Mg$^2+$ influx. HCO$_3^-$ may activate the Mg$^2+$ uptake system cooperatively or 2 HCO$_3^-$ may be cotransported with Mg$^2+$ in an Mg$^2+$,HCO$_3^-$ symport.

To characterize the action of HCO$_3^-$ in Mg$^2+$ uptake, DIDS and SITS, which inhibit HCO$_3^-$/Cl$^-$ exchange [7] and Na$^+$/HCO$_3^-$/Cl$^-$ exchange [5], were tested. As shown in fig.5, DIDS and SITS had no influence on Mg$^2+$ uptake. Thus, HCO$_3^-$/Cl$^-$ exchange and Na$^+$/HCO$_3^-$/Cl$^-$ exchange are not involved in Mg$^2+$ uptake.

A further differentiation of the involved transport system is indicated by the result that Mg$^2+$ uptake was not changed when extracellular Cl$^-$ was omitted (fig.5). Thus, Mg$^2+$ influx via the Na$^+$/K$^+$/Cl$^-$ cotransport which depends on extracellular Cl$^-$ cannot take place. As incubation in Cl$^-$-free medium depletes cells of intracellular Cl$^-$ [5], Mg$^2+$ uptake via Na$^+$/HCO$_3^-$/Cl$^-$ exchange [5] can also be excluded in agreement with the lack of effect of DIDS and SITS. Therefore, it may be suggested that Mg$^2+$ is taken up by Mg$^2+$,HCO$_3^-$ cotransport. The absence of an effect of DIDS and SITS on Mg$^2+$ influx is no argument against separate Mg$^2+$,HCO$_3^-$ cotransport. The inhibitory effect of DIDS and SITS may only be concerned with the exchange of HCO$_3^-$ against Cl$^-$ and not with Mg$^2+$,HCO$_3^-$ cotransport. Furthermore, the amount of Mg$^2+$ taken up was only 3 mmol/l cells within 2–3 h. The corresponding amount of HCO$_3^-$ taken up by Mg$^2+$,HCO$_3^-$ cotransport may not influence the rate of Mg$^2+$ uptake or could be eliminated by reactions other than HCO$_3^-$/Cl$^-$ exchange.

As another hint for the stoichiometry of Mg$^2+$ influx we tested whether Mg$^2+$ uptake depends on membrane potential. Depolarization of the cell membrane by incubation of cells in K$^+$ medium did not reduce Mg$^2+$ influx (fig.5), indicating an electroneutral uptake mechanism. Thus, our results can be interpreted by the assumption that Mg$^2+$ influx operates via electroneutral Mg$^2+$,HCO$_3^-$ symport, cotransporting 1 Mg$^2+$ and 2 HCO$_3^-$ into the cell.

Net Mg$^2+$ uptake in Mg$^2+$-depleted cells stopped when the original Mg$^2+$ content was achieved ([1] and figs 2,5). Probably, net Mg$^2+$ uptake via Mg$^2+$,HCO$_3^-$ cotransport is inhibited at normal intracellular Mg$^2+$ concentration.

ACKNOWLEDGEMENT

We thank R. Förster for her excellent technical assistance.
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