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

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Partially Mg^{2+}-depleted Yoshida ascites tumor cells took up Mg^{2+} after reincubation in Mg^{2+}- and HCO_3^--containing media. Mg^{2+} influx was insensitive to ouabain, amiloride and disulfonic stilbenes, but was non-competitively inhibited by furosemide ($K_i = 0.4$ mM) and bumetanide. Mg^{2+} influx obeyed Michaelis-Menten kinetics with respect to Mg^{2+} concentration ($K_m = 1.1$ mM) and was sigmoidal with respect to HCO_3^- concentration. Electroneutral Mg^{2+}, HCO_3^- cotransport was supposed to be the mechanism of Mg^{2+} influx.

Mg^{2+} influx *Furosemide sensitivity* *(Yoshida ascites tumor cell)*

1. INTRODUCTION

In [1], we described that thymocytes and ascites tumor cells, partially depleted of Mg^{2+}, take up Mg^{2+} after reincubation in Mg^{2+}-containing media depending on the presence of HCO_3^-. Here, we characterize the Mg^{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 $NaHCO_3$, 0.1 g/l streptomycin and 0.06 g/l penicillin.

For Mg^{2+} depletion, the ascites tumor cells (cell concentration 1%) were incubated at 37°C for 1.5 h in Na^+ medium with 5 mM 2-DOG instead of 5 mM glucose. 2-DOG was phosphorylated by ATP resulting in ATP depletion and Mg^{2+} efflux [1]. The Na^+ medium contained (in mM): 145 NaCl, 5 KCl, 1 Na_2HPO_4, 0.5 $MgCl_2$, 1.2 $CaCl_2$, 30 Hepes, pH 7.4, 5 glucose. For removal of 2-DOG the cells were centrifuged at 1000 × *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_3^--containing medium was used for reincubation, the medium was gassed with air-5% CO_2. Some experiments were done with K^+ medium, containing (in mM): 120 KCl, 20 $KHCO_3$, 1 K_2HPO_4, 0.5 $MgCl_2$, 1.2 $CaCl_2$, 30 Hepes, pH 7.4, 5 glucose and with Cl^--free medium, containing (in mM): 50 $NaHCO_3$, 0.5 Mg acetate, 1 Na_2HPO_4, 5 glucose and 150 sucrose. The viability of the cells during Mg^{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^{2+} was

Abbreviations: 2-DOG, 2-deoxyglucose; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid; $[Mg^{2+}]_i$, intracellular Mg^{2+} content; $[Mg^{2+}]_o$, extracellular Mg^{2+} concentration

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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

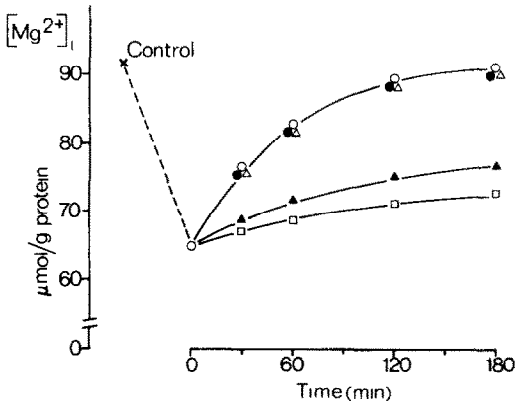

Fig.1. Re-uptake of Mg^{2+} in Mg^{2+}-depleted Yoshida ascites tumor cells. Cells were reincubated in Na^+ medium with: 20 mM HCO_3^- (○), 20 mM HCO_3^- and 0.1 mM ouabain (●), 20 mM HCO_3^- and 1 mM amiloride (△), 20 mM HCO_3^- and 1 mM furosemide (□), 20 mM HCO_3^- and 1 mM bumetanide (▲). Mean of 3 experiments.

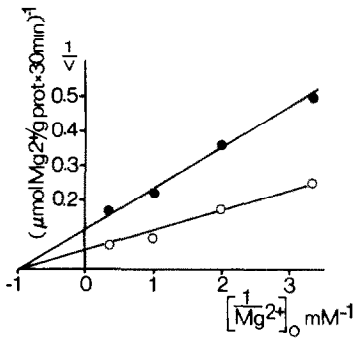

Fig.2. Double-reciprocal plot of Mg^{2+} influx ($1/v$) vs $1/[Mg^{2+}]_o$. Mg^{2+}-depleted Yoshida ascites tumor cells were reincubated in Na^+ medium with 20 mM HCO_3^- and different Mg^{2+} concentrations. Incubation without furosemide (○), in the presence of 0.5 mM furosemide (●).

Na^+,K^+,Cl^- cotransport [6] and $Na^+,HCO_3^-/Cl^-$ exchange [5].

Mg^{2+} influx was dependent on HCO_3^- [1]. Fig.3 shows that the Mg^{2+} influx dependence on HCO_3^- concentration gives rise to a sigmoidal curve and furosemide inhibits Mg^{2+} influx by reducing the velocity of Mg^{2+} uptake.

When the values of fig.3 were plotted as a function of $\log[HCO_3^-]$ vs $\log v/V_{max} - v$ (Hill plot [8]), where v is the rate of Mg^{2+} influx at a distinct HCO_3^- concentration and V_{max} the maximal rate of

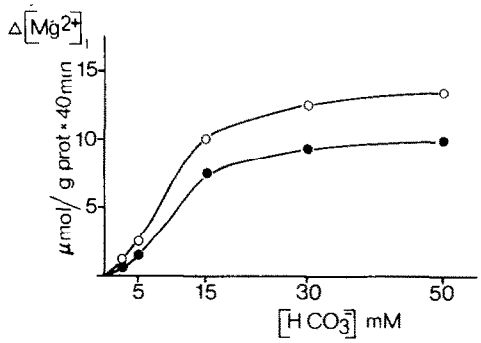

Fig.3. Rate of intracellular Mg^{2+} uptake in Mg^{2+}-depleted Yoshida ascites tumor cells. Cells were incubated in Na^+ medium with different HCO_3^- concentrations. Incubation without furosemide (○), in the presence of 0.3 mM furosemide (●). Mean of 3 experiments. The pH of the medium with 2.5, 5, 15, and 30 mM HCO_3^- amounted to 7.40; pH in the medium with 50 mM HCO_3^- amounted to 7.46.

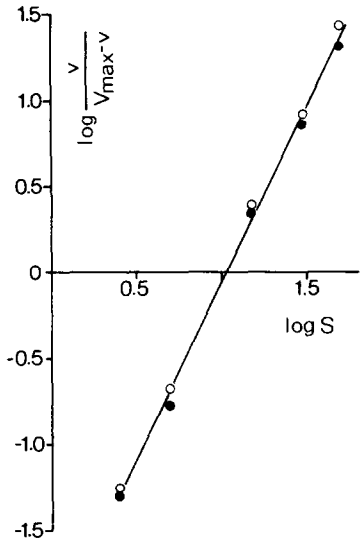

Fig.4. Values of fig.3 plotted according to Hill [8] as a function of log $v/V_{max} - v$ vs log S (log[HCO_3^-]).

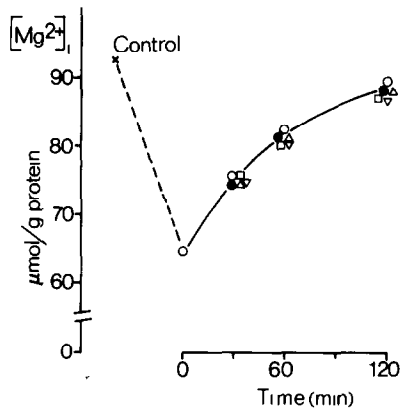

Fig.5. Re-uptake of Mg^{2+} in Mg^{2+}-depleted Yoshida ascites tumor cells after reincubation in Na^+ medium with: 20 mM HCO_3^- (○), 20 mM HCO_3^- and 1 mM DIDS (●), 20 mM HCO_3^- and 1 mM SITS (△), K^+ medium (□) and Cl^- free medium (▽). Mean of 2 experiments.

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.

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