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Regulation of intracellular magnesium by Mg^{2+} efflux

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Summary: Chicken erythrocytes were loaded with Mg^{2+} by incubation with the cation ionophore A 23187 in the presence of Mg^{2+}. After removing A 23187 by intensive washing with serum albumin and reincubating the Mg^{2+}-loaded cells, Mg^{2+} was transported out of the cells until the original Mg^{2+} content was achieved. The net Mg^{2+} efflux followed Michaelis-Menten-kinetics and was independent of extracellular and intracellular Ca^{2+} and calmodulin. The net Mg^{2+} efflux was not affected by adrenalin, isoproterenol, p-chloromercuribenzenesulfonate, ouabain and tetrodotoxin, but was inhibited by dicyclohexylcarbodiimide, KCN, iodoacetate, high extracellular concentrations of Mg^{2+}, Mn^{2+} and when extracellular Na^{+} was substituted by choline or K^{+}. The efflux of 1 Mg^{2+} was coupled with the uptake of 2 Na^{+}. It is concluded that there exists an additional gating process at the inner cell surface becoming active only at increased concentrations of intracellular free Mg^{2+} regulating the exit of Mg^{2+} by the efflux system.

Several experimental results have indicated that the concentration of intracellular free Mg^{2+} remained constant, although the total Mg^{2+} content was changed. By incubating ascites tumor cells with adenosine, cellular ATP concentration and thereby MgATP and total Mg^{2+} were increased, whereas the free Mg^{2+} concentration remained constant (1). When the cellular ATP concentration was reduced, e.g. in cardiac cells, either by ischaemia (2) or by metabolic inhibitors (3), again, the intracellular free Mg^{2+} concentration remained constant. From these results it can be concluded that when cellular ATP is reduced and the cytoplasm is acidified, the intracellular free Mg^{2+} concentration is transiently increased and consequently Mg^{2+} is transported out of the cell until the physiological value is achieved. In the present study, this hypothesis was investigated and a description of the mechanism is given.

Materials and Methods

Blood was taken from chicken (strain Warren SSL) by vein puncture in heparinized centrifuge tubes, centrifuged at 1,000 g for 5 min at room temperature

Abbreviations: pCMBS, p-chloromercuribenzenesulfonate; DCCD, dicyclohexylcarbodiimide; TCA, trichloroacetic acid.

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and washed twice in K^+ medium, containing (in mM): 140 KCl, 10 NaCl, 6 $MgCl_2$, 1 Na_2HPO_4, 5 glucose, 30 TrisCl, pH 7.4, 50 sucrose, 1.2 $CaCl_2$ present as indicated in Table 1. The erythrocytes were loaded with Mg^{2+} by incubating a 10 % cell suspension in K^+ medium with 6 µM A 23187 (dissolved in DMSO) for 15 min at 37 °C. For the removal of A 23187, the cells were centrifuged and reincubated 3 times for 5 min at 37 °C in K^+ medium with 1 % bovine serum albumin and washed twice in 150 mM NaCl/50 mM sucrose at 0 °C.

For measuring the Mg^{2+} efflux the cells were incubated as a 10 % suspension in a medium containing one third chicken plasma and two thirds Na^+ medium at 37 °C. The Na^+ medium contained (in mM): 145 NaCl, 5 KCl, 1 Na_2HPO_4, 1.2 $CaCl_2$, 5 glucose, 2 pyruvate, 30 TrisCl, pH 7.4, 50 sucrose.

For experiments in the Ca^{2+}-free medium, chicken plasma and $CaCl_2$ were omitted. For experiments in the Na^+-free medium, NaCl was substituted by choline chloride.

After various incubation times as indicated, 1 ml cell suspension was centrifuged for 1 min at 10,000 g in an Eppendorf centrifuge. For Mg^{2+} determination, 2 ml 10 % TCA/0.1 % La^{3+} were added to 100 µl of the supernatant. The cells were washed twice at 0 °C in 150 mM NaCl/50 mM sucrose. For the determination of intracellular Na^+, the cells were washed with 150 mM choline chloride/50 mM sucrose. The washed cells were hemolysed by adding 750 µl H_2O. 50 µl were taken for the determination of hemoglobin according to (4). The rest was deproteinized by addition of 50 µl 75 % TCA and centrifuged. 100 µl of the supernatant were diluted with 2 ml 10 % TCA/0.1 % La^{3+} for the determination of Mg^{2+}. Mg^{2+} was measured by atomic absorption spectrophotometry (Perkin-Elmer, model 303). Na^+ was measured in the deproteinized supernatant after the addition of LiCl by flame photometry (Beckman). Bovine serum albumin was obtained from Serva, Heidelberg, A23187 from Boehringer, Mannheim, all other reagents (analytical grade) from Merck, Darmstadt, (FRG).

Results and Discussion

Removal of A 23187: Demonstration of the cell membrane by scanning electron microscopy, intracellular K^+ and Na^+ concentration, and hemolysis were not good indicators for complete removal of A 23187, because they were the same for incomplete and complete removal of A 23187. Complete removal of A 23187 was proved in the following way. Chicken erythrocytes were depleted of Mg^{2+} by adding A 23187 and EDTA. After insufficient washing, due to remaining A 23187, the cells took up Mg^{2+} when reincubated in an Mg^{2+}-containing medium. After intensive washing according to the used procedure, the Mg^{2+}-depleted cells did not take up Mg^{2+} after reincubation.

Kinetics of the net Mg^{2+} efflux: As shown in Fig. 1, the cells can be loaded with Mg^{2+} by incubation with the cation ionophore A 23187 in the presence of Mg^{2+}. After removing the membrane-bound ionophore by intensive washing the cells with serum albumin and after reincubating the cells, Mg^{2+} is transported out of the cells, as can be seen by the decrease in cellular Mg^{2+} content. Net Mg^{2+} efflux stops when the original Mg^{2+} content is reached. Also

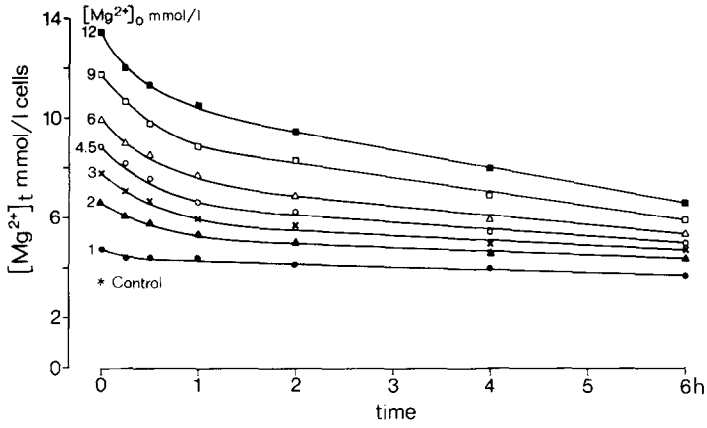

Fig. 1. Intracellular Mg^{2+} content ($[Mg^{2+}]_t$) of Mg^{2+}-loaded chicken erythrocytes at various times after reincubation. The cells were preloaded with Mg^{2+} by incubation with A 23187 at various extracellular Mg^{2+} concentrations ($[Mg^{2+}]_o$) as indicated. Control: Mg^{2+} content of untreated erythrocytes.

by long-term incubation (up to 18 hours) or by incubation in Mg^{2+}-free or EDTA-containing media, cellular Mg^{2+} content is not reduced below the Mg^{2+} content of the control cells (not shown).

The velocity of net Mg^{2+} efflux can be shown more precisely by measuring the increase of extracellular Mg^{2+} concentration during the net Mg^{2+}efflux (Fig.2, same experiment as in Fig.1). The rate of the net Mg^{2+} efflux is increased when cellular Mg^{2+} is increased.

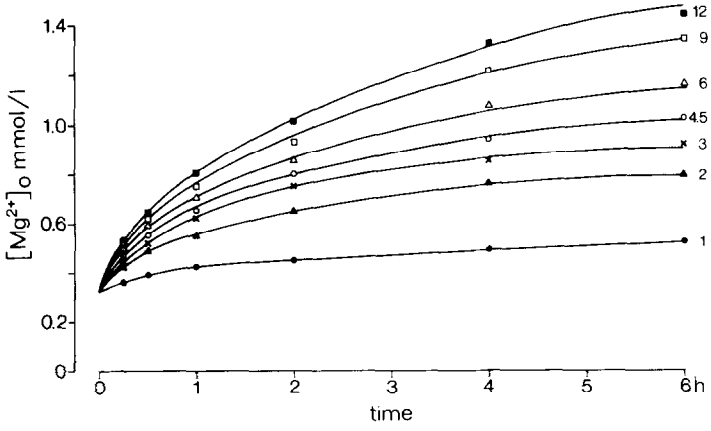

Fig. 2. Increase of extracellular Mg^{2+} concentration during reincubation of Mg^{2+}-loaded chicken erythrocytes. Same experiment as in Fig. 1.

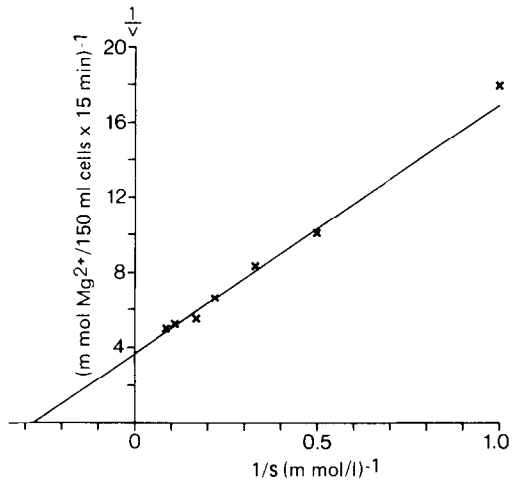

Fig. 3. Lineweaver-Burk plot of net Mg^{2+} efflux. Same experiment as in Figs. 1 and 2. Values for v and S were taken from Fig. 2. For details see text.

According to Flatman and Lew (5) A 23187 produces an equilibration of extracellular and intracellular concentration of free Mg^{2+}. Therefore, from the experimental data contained in Fig.2, a double reciprocal plot of 1/v (1/rate of net Mg^{2+} efflux) vs. 1/S (1/intracellular free Mg^{2+} concentration) can be performed (Fig. 3). The kinetic analysis of this experiment resulted in Michaelis-Menten-kinetics (K_m = 3.5 mmol/l), indicating the involvement of a rate-limiting step at the cell membrane.

Properties of the net Mg^{2+} efflux: A net Mg^{2+} efflux from heart muscle cells can be induced by injecting isoproterenol (6, 7). At the same time, the Ca^{2+} content in the heart increases (7, 8). Since an increase in intracellular Ca^{2+} concentration can induce a net K^+ efflux (Gardos-effect) (9), the influence of Ca^{2+} on net Mg^{2+} efflux was tested (Table 1).

Preloading the cells with Mg^{2+} in the absence or presence of extracellular Ca^{2+}, causing a simultaneous Ca^{2+}-loading, resulted in the same net Mg^{2+} efflux.

The presence or absence of extracellular Ca^{2+} during net Mg^{2+} efflux did not have any influence on net Mg^{2+} efflux either. As the intracellular Ca^{2+} is strongly compartmentalized and the behaviour of cytoplasmic free Ca^{2+} concentration is unknown under our conditions, the influence of the Ca^{2+}calmodulin

Table 1

Addition (mM)	$[Ca^{2+}]_o$ during preloading (mM)	$[Ca^{2+}]_o$ during reincubation (mM)	Net Mg^{2+} efflux (% of control)
-	0.5	1.2	100
-	0	1.2	100
-	0	0	100
Trifluoperazine, 0.2	0.5	1.2	105
Trifluoperazine, 0.2	0	1.2	105
Adrenalin, 0.1	0	1.2	104
Isoproterenol, 0.01	0	1.2	103
PCMBS, 0.1	0	1.2	97
DCCD, 1	0	1.2	30
KCN, 1	0	1.2	85
Iodoacetate 1	0	1.2	45
KCN, 1; iodoacetate, 1	0	1.2	20
K^+, 140	0	0	25
Choline, 145	0	1.2	10
Ouabain, o.1	0	1.2	96
Tetrodotoxin, 0.1	0	1.2	103
$[Mn^{2+}]_i$ 0.4	0	1.2	15
$[Mn^{2+}]_o$ 0.01	0	1.2	70
0.1	0	1.2	60
$[Mg^{2+}]_o$ 0.3	0	1.2	100
1	0	1.2	85
3	0	1.2	65
10	0	1.2	40
30	0	1.2	0

Net Mg^{2+} efflux from chicken erythrocytes. The cells were loaded with Mg^{2+} by incubation with 3 µM A 23187 in the presence of 6 mM Mg^{2+}. After removal of A 23187, the cells were reincubated in Na^+ medium with various additions as indicated. When pCMBS and DCCD were tested, the cells were preincubated with the inhibitors for 30 min at 37 °C before the net Mg^{2+} efflux was measured. $[Mn^{2+}]_i$ means: after Mg^{2+} preloading, the cells were additionally loaded with Mn^{2+} by adding 0.4 mM $MnCl_2$.

antagonist trifluoperazine was examined. Again, after preloading the cells with Mg^{2+} in the presence or absence of Ca^{2+}, trifluoperazine had no effect on the net Mg^{2+} efflux. Therefore, Ca^{2+} is not involved in net Mg^{2+}efflux. Moreover, adrenalin and isoproterenol have no influence on the net Mg^{2+} efflux in chicken erythrocytes.

To establish whether proteins are involved in the net Mg^{2+} efflux, group-specific inhibitors were added (Table 1). PCMBS reacting with SH-groups was ineffective. However, DCCD reacting with carboxyl-, tyrosyl-, or seryl-groups of proteins e.g. of the proton-conducting channel (10) inhibited the net Mg^{2+} efflux, indicating the involvement of membrane-bound proteins in the Mg^{2+} efflux.

As can be seen from the inhibition by KCN and iodoacetate (Table 1), the net Mg^{2+} efflux is energy-dependent. Energy may be required to maintain the membrane potential, or ATP may produce a phosphorylation or an allosteric transition of a cell membrane component. The net Mg^{2+} efflux was reduced in a medium with high K^+ concentration (Tab. 1). However, this effect cannot be explained by a depolarization of the cell membrane. As the inside of the cell membrane is negatively charged, a reduction of the membrane potential would favour the net Mg^{2+} efflux. Therefore, the inhibition of the net Mg^{2+} efflux in a high K^+ medium may reflect the specific dependency of the Mg^{2+} efflux by extracellular Na^+. Indeed, substitution of NaCl by choline chloride in the efflux medium reduced the net Mg^{2+} efflux drastically (Table 1). From these results it can be concluded that the net Mg^{2+} efflux is coupled to Na^+ uptake.

The stoichiometric relationship of the net Mg^{2+} efflux with the net Na^+ influx is shown in Fig. 4. During the first 30 min of the Mg^{2+} efflux, the cellular Mg^{2+} content was reduced by 1.5 mmol/l cells, whereas at the same time the cellular Na^+ content was increased by 3 mmol/l cells. This demonstrates an exchange of 1 Mg^{2+} for 2 Na^+. After that time, the net intracellular Na^+ uptake is lower because of the net Na^+ transport out of the cells. On inhibition of the active Na^+ transport by ouabain, the Na^+/Mg^{2+} ratio becomes higher which is probably due to passive diffusion of Na^+ into the cell.
As only a small amount of Na^+ is taken up by the cells and as the uptake of Na^+ proceeds slowly, it can be explained that the net Mg^{2+} efflux is not inhibited by ouabain or by tetrodotoxin that blocks fast Na^+ channels of either

high or low affinity to tetrodotoxin (Table 1). However, it is still unknown whether chicken erythrocytes possess fast Na^+ channels.

Mn^{2+} inhibits the net Mg^{2+} efflux at the inner cell membrane when the cells are subsequently loaded with Mn^{2+}. (Simultaneous preloading with Mg^{2+} and Mn^{2+} is not possible because Mn^{2+} inhibits preloading with Mg^{2+} when both ions are added simultaneously.)

Mn^{2+} also inhibits the net Mg^{2+} efflux at the outer cell membrane when added to Mg^{2+}-preloaded cells (after removal of A 23187).

Therefore, the effect of elevated extracellular Mg^{2+} on the net Mg^{2+} efflux was tested. The increase of the extracellular Mg^{2+} concentration to 30 mmol/l inhibited net Mg^{2+} efflux completely (Table 1). This result may be attributed to an inhibition or shift of the equilibrium of a reversible reaction at the outside of the cell membrane by high extracellular Mg^{2+}.

When discussing the mechanism of the net Mg^{2+} efflux one has to consider that the net Mg^{2+} efflux is only operating at increased intracellular free Mg^{2+} concentration. Having reached the physiological level of free Mg^{2+} in the cells, the net Mg^{2+} efflux stops. Such a strong Mg^{2+} dependency cannot be fulfilled by a simple Mg^{2+} efflux mechanism obeying Michaelis-Menten-kinetics. Therefore, a second mechanism must exist, representing an additional gating

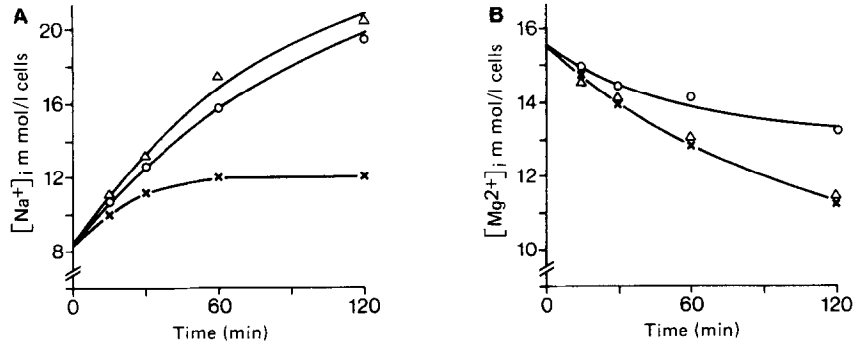

Fig. 4 A. Increase of intracellular Na^+ concentration ($[Na^+]_i$) in Mg^{2+}-loaded chicken erythrocytes after removal of A 23187 and reincubation. x, control ; Δ, reincubation with 0.1 mM ouabain, o, reincubation with 1 mM KCN + 1 mM iodoacetate.

Fig. 4 B. Decrease of intracellular Mg^{2+} content ($[Mg^{2+}]_i$) of Mg^{2+}-loaded chicken erythrocytes. Same experiment as in Fig. 4 A.

process that becomes active only at increased intracellular free Mg^{2+}, allowing the exchange of 1 Mg^{2+} for 2 Na^{+} by the efflux system.

Whether ATP or the DCCD-inhibited protein are involved in the gating or in the efflux process is unknown. ATP may operate directly by phosphorylation or allosterically. An indirect effect of ATP by maintaining the Na^{+}-gradient, can be excluded, because ouabain produced the same increase in intracellular Na^{+} concentration as inhibition of ATP synthesis, but no inhibition of net Mg^{2+} efflux (Fig. 4).

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