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UPTAKE OF Mg^{2+} BY KB CELLS

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SUMMARY

Suspension cultures of KB cells accumulate $^{28}Mg^{2+}$ by a transport system which is saturable and specific. At 37° the uptake of $^{28}Mg^{2+}$ is approximately linear for 1–2 h and follows standard Michaelis–Menten kinetics with a $K_m = 0.1$ mM Mg^{2+} and a $v_{max} = 0.4$ μmole/h per $1 \cdot 10^8$ cells. The $^{28}Mg^{2+}$ which has been taken up by the cells is exchangeable with Mg^{2+} added after 5 h exposure and can be released by agents which cause membrane leakiness. Inhibitors such as cyanide, *m*-chlorophenyl carbonylcyanide hydrazone (CCCP) and ouabain reduce the uptake of Mg^{2+} by 50 % or more.

INTRODUCTION

Although Mg^{2+} is the most common bivalent cation in cell cytoplasm, there have been no reports of the existence of a specific Mg^{2+} transport system in animal cells. There has been much work done on net Mg^{2+} fluxes in various systems, particularly yeast cells[1], but transport studies have been hampered by lack of purified, high specific activity $^{28}Mg^{2+}$ (refs. 2 and 3). However, $^{28}Mg^{2+}$ in a suitable form has recently become available[4], and its use has led to the description of a specific Mg^{2+} active transport system in *Escherichia coli*[5,6]. In this first report, the general characteristics of the uptake and exchange of $^{28}Mg^{2+}$ by human KB cells in suspension culture are described.

MATERIALS AND METHODS

Cells and media

KB cells were obtained from Dr. H. Raskas, St. Louis University School of Medicine, and maintained in suspension cultures at 37° in minimal essential medium (Joklik-modified, GIBCO), with 10 % horse serum added. This medium contains 1 mM Mg^{2+} as measured by atomic absorption spectroscopy. The cells were harvested after growth to $2.5 \cdot 10^5$–$5 \cdot 10^5$ cells/ml by centrifugation for 5 min at 350 × *g* in conical glass tubes in a Model PR-2 International centrifuge, washed once with a balanced salt solution from which Mg^{2+} was omitted, and resuspended in spinner medium, also without Mg^{2+}. The low Mg^{2+} balanced salt solution contained 110 mM NaCl, 5.4 mM KCl, 9.6 mM NaH_2PO_4, 11 mM glucose, and 2.4 mM $NaHCO_3$. For

Abbreviation: CCCP, *m*-chlorophenyl carbonylcyanide hydrazone.

low Mg^{2+} spinner medium, the balanced salt solution was supplemented with Eagle's basal medium vitamins (100 ×) and Eagle's basal medium minerals (100 ×) (Hyland, 10 ml of each per l), penicillin and streptomycin (GIBCO, 100 units/ml and 100 μg/ml), 2 mM glutamine, and 10 % dialysed horse serum (GIBCO). While no Mg^{2+} was added to this medium, 0.01 mM Mg^{2+} (measured by atomic absorption spectroscopy) was present from contamination of the other ingredients. Cells incubated in low Mg^{2+} medium for periods up to 9 h appeared normal under the phase contrast microscope. Membrane integrity, as indicated by the ability of cells to exclude trypan blue dye[7] was not affected.

Radioisotope and counting

$^{28}Mg^{2+}$ ($t_{1/2}$ = 21.3 h) was obtained from the Brookhaven National Laboratory, Upton, New York, and added to the cultures at about 0.1 μC/ml.

The suspension of cells was maintained at 37° with gentle agitation during exposure to $^{28}Mg^{2+}$. 1-ml samples were filtered rapidly on Millipore AA filters (0.8-μ pore size) and washed twice with 5 ml each of Mg^{2+}-free salt solution at 25 or 37°. Additional washing or washing with balanced salt solution at 4° did not appreciably alter the amount of radioactivity retained on the filters.

In different experiments, three methods of counting radioactivity on the filters have been used, all of which are quite satisfactory: 1. Filters were glued onto planchets and counted in a Nuclear Chicago gas-flow counter with a 50 % efficiency. 2. Filters were dissolved in 10 ml of a liquid scintillation cocktail containing dioxane and naphthalene[8] and counted in a Packard liquid scintillation spectrometer Model 3375 with the preset ^{32}P channel. 3. Filters were placed in 10 ml water and counted in the Packard spectrometer with a 20 % gain setting and 50–1000 window *via* Cerenkov radiation[9]. The count rate for similar samples was about twice as high by liquid scintillation counting as on the gas-flow counter and approximately equal on the gas-flow counter and by Cerenkov counting in the scintillation spectrometer. Direct 0.1-ml samples from the radioactive cultures were also counted for $^{28}Mg^{2+}$.

Cell numbers were determined with a white blood cell counter (C. A. Hauser Company) under a phase contrast microscope. A cell count of $3 \cdot 10^5$ cells/ml corresponds to a packed volume of 0.2 % (cell volume/medium volume) as determined by centrifugation in an A. H. Thomas thrombocytocrit tube. The Mg^{2+} content of cells was determined by washing twice with low Mg^{2+} balanced salt solution, concentrating in distilled water, digesting with acid or detergent and assaying with a Perkin–Elmer 303 atomic absorption spectrometer. KB cells grown in spinner medium containing 1 mM Mg^{2+} accumulate approx. 2 μmoles Mg^{2+} per $1 \cdot 10^8$ cells. (This would correspond to an upper limit of 3 mM internal Mg^{2+} based on the assumption that all of the intracellular Mg^{2+} is free and not bound.)

Solapalmitine[10] was a gift of Dr. S. M. Kupchan. Ouabain was obtained from Sigma Chemical Company, St. Louis, and CCCP from Calbiochem, Los Angeles.

RESULTS

$^{28}Mg^{2+}$ uptake

When $^{28}Mg^{2+}$ is added to KB cells in suspension culture the amount of radioactivity taken up by the cells depends, as expected, on whether they are suspended

in the normal high Mg^{2+} growth medium or in a medium with a lower Mg^{2+} content (Fig. 1). The $^{28}Mg^{2+}$ uptake in the low Mg^{2+} medium is reasonably consistent with "two compartment exchange kinetics"[11] and when plotted as log $[1-^{28}Mg^{2+}(t)/^{28}Mg^{2+}(\infty)]$ *vs.* time (t) in hours, one obtains a straight line with a half-equilibrium time of 3.3 h (Fig. 1). After 5 h incubation with 0.02 mM $^{28}Mg^{2+}$, the Mg^{2+} concentration of an aliquot was raised to 1.2 mM. More than 75 % of the $^{28}Mg^{2+}$ which had been taken up by the cells was released, presumably by exchange of nonradioactive Mg^{2+} for $^{28}Mg^{2+}$.

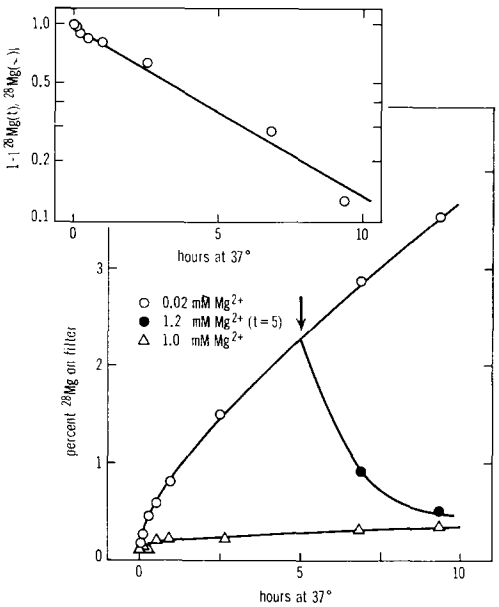

Fig. 1. Uptake of $^{28}Mg^{2+}$ in high and low Mg^{2+} media. KB cells were incubated at 37° in either normal 1.0 mM Mg^{2+} growth medium or in low Mg^{2+} medium in which a principal source of Mg^{2+} was the 0.01 mM $^{28}Mg^{2+}$ added at time 0. After 5 h, the low Mg^{2+} culture was divided and 1.2 mM Mg^{2+} was added to one portion. All points except those at 2 min represent the average of two values. In the inset, the uptake of $^{28}Mg^{2+}$ is plotted according to the equation for two compartment exchange kinetics: log $[1-^{28}Mg^{2+}(t)/^{28}Mg^{2+}(\infty)] = kt$. 4 % was assumed as an approximate value for the $^{28}Mg^{2+}$ in the cells at equilibrium ($t = \infty$) by best fit of the equation with the data in Fig. 1.

Since both active transport and facilitated diffusion involve specific carrier systems and differ from passive diffusion in that the carrier molecules are saturated in the presence of high concentrations of substrate, the effect of increasing Mg^{2+} concentration on uptake was tested more carefully. The results of such an experiment are given in Fig. 2; the initial rates, treated according to LINEWEAVER AND BURK[12] in Fig. 2a, show saturation kinetics with a $K_m = 0.11$ mM Mg^{2+} and a $v_{max} = 0.4$ μmole/h per $1 \cdot 10^8$ cells. Although a smaller fraction of the higher concentration of Mg^{2+} was accumulated, this represents a greater total uptake of Mg^{2+} (compare Figs. 1 and 2). The lines drawn for the initial rates of uptake of higher concentrations of $^{28}Mg^{2+}$ in Fig. 2b do not extrapolate to the abscissa at 0 time, but rather there seems to be a rapid initial uptake of a small fraction of $^{28}Mg^{2+}$ followed by the linear uptake shown in Fig. 2b. We have not determined whether this result is

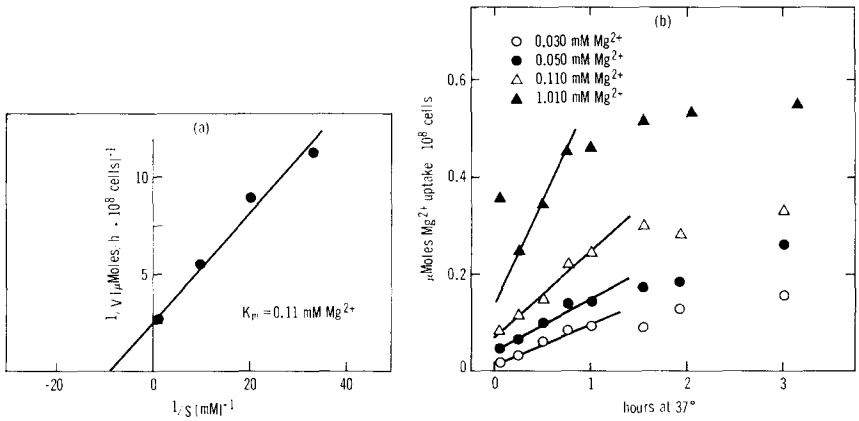

Fig. 2. Saturation kinetics of the Mg^{2+} transport system. $^{28}Mg^{2+}$ uptake was followed for a 3-h period with KB cells exposed to 0.03–1.01 mM Mg^{2+}. As opposed to the data analysis in Fig. 1, here the fractional incorporation of $^{28}Mg^{2+}$ was multiplied by the specific activity to give μmoles Mg^{2+} uptake per $1 \cdot 10^8$ cells. The rate of uptake of $^{28}Mg^{2+}$, as indicated in (b), is plotted in (a) according to LINEWEAVER AND BURK[12]. These data are from one of three separate experiments, all with comparable results.

indicative of a small surface "compartment" with more rapid exchange, as has been invoked to explain such results[11,13,14], or reflects nonspecific binding to the cell surface or to the Millipore filter itself. Millipore binding, if it occurred, never exceeded 0.1 % of the $^{28}Mg^{2+}$ present and did not affect our studies on the major cell compartment.

Specificity and inhibition

The KB cell system for uptake of $^{28}Mg^{2+}$ is highly specific. Whereas the uptake system is half saturated by 0.11 mM Mg^{2+}, the uptake of $^{28}Mg^{2+}$ in 0.02 mM Mg^{2+} is unaffected by the addition of 1.0 mM Ca^{2+} or 0.1 mM Mn^{2+}. Mn^{2+} at 1 mM immediately inhibits $^{28}Mg^{2+}$ uptake by 80 %. However, this concentration of Mn^{2+} is cytotoxic over a prolonged period of time and thus the directness of its effect on Mg^{2+} transport is uncertain.

In an attempt to further characterize the Mg^{2+} uptake system, effects of various energy inhibitors were investigated. In the experiment shown in Fig. 3a we tested cyanide, presumably a general energy poison acting at the level of the cytochromes, and CCCP, one of the more potent of the new "uncouplers" of oxidative phosphorylation. Both inhibited $^{28}Mg^{2+}$ uptake by about 50 %. Although this degree of inhibition does not permit a definite distinction between (energy-requiring) active transport and (non-energy requiring) facilitated diffusion[13], it should be noted that inhibitions of 25–50 % have been considered evidence for active transport in other mammalian cell and tissue studies[11,15–17]. Also tested in the same experiment (Fig. 3a) were ouabain, a cardiac glycoside which inhibits the (Na^+-K^+)-ATPase responsible for alkali cation active transport[18] and solapalmitine, a plant alkaloid, which causes non-specific loss of small molecules without directly affecting transport[19,20] Both of these latter compounds were more inhibitory than cyanide or CCCP. The action of ouabain can be explained either by direct inhibition of a specific Mg^{2+} transport

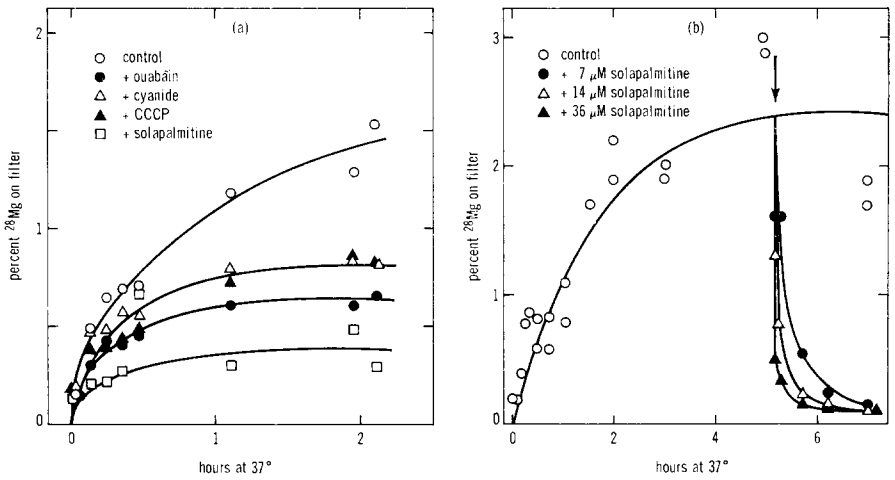

Fig. 3. Effects of inhibitors on the accumulation of $^{28}Mg^{2+}$. (a). 5 min before the addition of 0.1 μC/ml $^{28}Mg^{2+}$, 0.02 mM ouabain, 10 mM sodium cyanide, 0.1 mM CCCP or 0.1 mM solapalmitine were added to the indicated cultures. (b). After 5 h incubation with 0.1 μC/ml $^{28}Mg^{2+}$, the culture was divided and solapalmitine added at the concentrations indicated. All cultures contained approx. 0.015 mM total Mg^{2+}.

protein or indirectly by action *via* the $(Na^+\text{-}K^+)$-ATPase. As yet we cannot distinguish between these two possibilities. Low concentrations of solapalmitine cause rapid loss of already accumulated $^{28}Mg^{2+}$ (Fig. 3b). Since an agent, which causes nonspecific leakiness[19,20] and therefore a great increase in the rate of passive diffusion in and out of the cells, causes a loss rather than an accelerated uptake of $^{28}Mg^{2+}$, we can conclude again that passive diffusion is not the basic mechanism for $^{28}Mg^{2+}$ uptake. Still another indication of the energy dependence of this uptake is that cells incubated at 27° take up $^{28}Mg^{2+}$ at 40 % the rate of 37° cells.

DISCUSSION

These preliminary experiments suggest that mammalian cells like some bacteria[5,6] have a specific transport system for Mg^{2+} uptake. The specificity is indicated by the discrimination between Mg^{2+}, Ca^{2+} and Mn^{2+} for uptake by the cells. Although our evidence at this stage is not sufficient to distinguish between facilitated diffusion and active transport for the mechanism of Mg^{2+} uptake, passive diffusion seems unlikely for three reasons: (1) Passive diffusion systems do not show saturation kinetics with clearly defined K_m's as do facilitated diffusion and active transport systems[13]. (2) There is no reason to expect energy inhibitors to alter the rate of passive diffusion. And (3) if Mg^{2+} were entering the cell by passive diffusion, the addition of solapalmitine which breaks down the cellular permeability barrier would accelerate the uptake. Exactly the opposite occurs and Mg^{2+} uptake is grossly inhibited by solapalmitine (Fig. 3a); $^{28}Mg^{2+}$ which has been previously accumulated by the cells leaks out when solapalmitine and other similar agents are added (Fig. 3b; ref. 20). While such leakage may also involve other substances of small molecular weight (*e.g.* possible Mg^{2+}-complexing agents like ATP) the fact that cells at low

(10 μM) solapalmitine concentrations appear normal by phase microscopy and continue to exclude trypan blue indicates that the cells do not suffer any gross damage. Since we cannot determine the fraction and nature of intracellularly bound Mg^{2+}, the inhibition of Mg^{2+} uptake by cyanide, CCCP and ouabain must be taken as suggestive of but inadequate to prove that there is a metabolically dependent Mg^{2+} transport system. Such a system could either be a primary active transport system or secondarily coupled to an active transport system. The extent of the ouabain sensitivity of Mg^{2+} uptake in KB cells (in *E. coli* and Euglena $^{28}Mg^{2+}$ accumulation is ouabain insensitive; unpublished data) makes it interesting to speculate that bivalent cation transport in mammalian cells might involve a (Mg^{2+}-Ca^{2+})-ATPase[15] system analogous to the mammalian cell (Na^+-K^+)-ATPase exchange system.

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