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Evidence that intracellular magnesium is present in cells at a regulatory concentration for protein synthesis

(growth regulation/DNA synthesis/potassium)

M. TERASAKI* AND H. RUBIN

Department of Molecular Biology, University of California, Berkeley, CA 94720

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ABSTRACT **When extracellular magnesium is reduced by a factor of 50 (from 1.0 to 0.02 mM), the total intracellular magnesium of a spontaneously transformed clone of 3T3 cells decreases by 30–50%. Protein synthesis rates in these cells were measured as the intracellular magnesium decreased. Protein synthesis rates and magnesium content were found to decrease in parallel with each other. At 3 hr, a decrease to 84% of control values of magnesium content was accompanied by a decrease to 85% of control values of leucine incorporation rates. A larger inhibition had occurred by 12 hr, when the magnesium had decreased to 67% and leucine incorporation rates had decreased to 57%. When magnesium was restored to magnesium-deprived cells, both magnesium content and leucine incorporation increased about 2-fold by 1 hr. In the experiments reported here, initial small changes in magnesium content are associated with changes in protein synthesis rates. This strongly suggests that magnesium is present at a regulatory rather than excess concentration for protein synthesis. The results are consistent with a role for intracellular magnesium in the regulation of protein synthesis and support the hypothesis that magnesium has a central role in the regulation of metabolism and growth.**

Protein synthesis rates in *in vitro* systems are known to be affected by magnesium (1). However, there has been little work devoted to characterizing the effects of magnesium on protein synthesis in intact cells. Perhaps the main reason is that magnesium is difficult to vary within cells—increases or decreases by a factor of 10 of extracellular magnesium have no effect on the total intracellular magnesium contents of the cell (2).

In an earlier paper, intracellular magnesium of 3T3 cells was varied by lowering the extracellular calcium concentration to 0.02 mM and then varying extracellular magnesium from 1 to 20 mM and to even higher inhibitory magnesium concentrations (3). A relationship of protein synthesis and intracellular magnesium was found that was similar to the "bell-shaped curve" of the dependence of protein synthesis on magnesium in *in vitro* systems. However, the need to lower the extracellular calcium complicates interpretation of the results.

A spontaneously transformed clone of 3T3 cells has been found that loses up to 50% of its total intracellular magnesium when the extracellular magnesium is lowered to 0.02 mM, even in the presence of a normal level of calcium (4). A study was undertaken to measure the rates of protein synthesis as this decrease in intracellular magnesium occurs.

MATERIALS AND METHODS

Cells. BALB/c 3T3 clone A31 cells were obtained from James Bartholomew. Spontaneously transformed cells were observed in the stock cultures after several months of passage. A clone of one of these spontaneously transformed cells was isolated and designated clone 14 (5). Since an aliquot was frozen and several thaws of this frozen stock have been used, the original continuously passaged population was named clone 14a (6). These were the cells used in the experiments.

Cells were grown in 60-mm polystyrene tissue culture dishes in incubators with 5% CO_2/95% air at 37°C.

Growth Medium. MCDB 402 (molecular, cellular, and developmental biology medium 402), a medium specially developed for 3T3 cells (7), was used. Cells were grown in 10% calf serum.

For the magnesium variation experiments, MCDB 402 was prepared without added magnesium. The residual magnesium was ≈0.01 mM, measured by atomic absorption; 10% dialyzed calf serum was used. It was prepared by dialyzing four times (12 hr for each change) against 30 vol of 150 mM sodium chloride. Before the changes to low magnesium medium, cultures were first washed twice with Tris-buffered saline in order to wash away magnesium remaining from the original medium.

Radioactive Labeling. [^{3}H]Leucine (original specific activity, 51.6 Ci/mmol; 1 Ci = 37 GBq) was used at an activity of 2 μCi/ml in 2 ml of medium for the 60-mm dishes. [^{3}H]Thymidine (original specific activity, 20 Ci/mmol) was used at an activity of 1 μCi/ml in 2 ml of medium. The length of the labeling period is given in the figure legends. All labeling media were made using medium of the same composition as the growth medium of the cells at the time of the label. At the end of the labeling period, cultures were washed three times with ice-cold Tris-buffered saline, extracted with ice-cold 5% trichloroacetic acid for 10 min, washed twice with trichloroacetic acid, and then solubilized in 2 ml of 0.2 M NaOH. Samples of the NaOH were taken for scintillation counting and protein determination. Duplicate protein determinations were run.

Ion Measurements. The method of Sanui and Rubin (8) was used. Briefly, cultures were first washed three times with CO_2-free 0.25 M sucrose (pH 7). This was followed by one wash with carbonated 0.25 M sucrose (pH 4), whose purpose was to displace externally bound cations by protons. The cells were scraped into distilled deionized water. Duplicate samples were taken for protein determination. Samples were also taken for determination of magnesium and potassium content. These samples were made to 15 mM La^{3+}/4 mM CsCl/100 mM HCl to minimize ionic and chemical interference, and then the ion concentrations were determined by using a Perkin-Elmer 402 atomic absorption spectrophotometer.

RESULTS

The spontaneously transformed clone 14a cells were changed into low magnesium (0.02 mM added) or normal magnesium

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*Present address: Department of Anatomy and Cellular Biology, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115.

(1.0 mM) medium. Total protein, thymidine, and leucine incorporation and total intracellular magnesium and potassium contents were determined at various times and are shown in Fig. 1.

The cell protein density at the start of this experiment was 75 $\mu g/cm^2$. An inhibition of growth was evident at 12 hr after magnesium deprivation (Fig. 1*a*). Leucine incorporation rates decreased to 85% of control rates at 3 hr, to 57% at 12 hr, and leveled off at a little more than 50% relative to control values (Fig. 1*b*). Intracellular magnesium content had similar kinetics: a decrease to 84% of control values at 3 hr, a decrease to 67% at 12 hr, and a leveling off, also with a little more than a 50% decrease relative to control values, at later times (Fig. 1*c*). Intracellular potassium contents relative to control values also decreased. The decreases were smaller: to 93% of control values at 3 hr, to 95% at 12 hr, and to ≈84% afterwards (Fig. 1*d*). There was a lag in the effect of low extracellular magnesium on thymidine incorporation. The thymidine incorporation rates began to decrease only after 12 hr. They decreased rapidly thereafter until there was an inhibition by a factor of ≈100 relative to control at 48 hr (Fig. 1*e*). This experiment was done three times with similar results each time.

The effects of the restoration of magnesium were determined. Clone 14a cells were plated and grown in the same manner as described above. The cells were changed to media

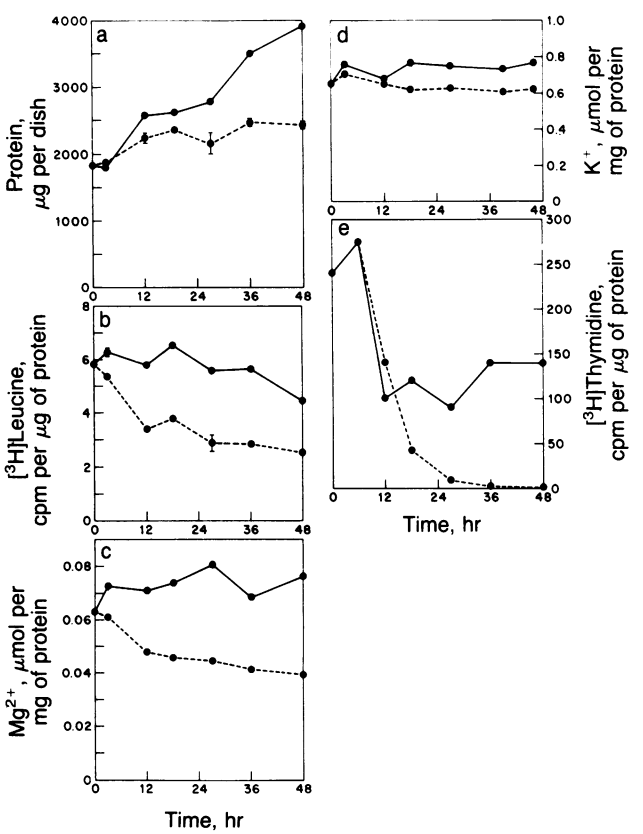

FIG. 1. Effects of magnesium deprivation on clone 14a cells. Clone 14a cells were seeded at 10^5 cells per 60-mm dish in medium containing 10% serum. The medium was replenished at 2 days. At 2.5 days, the medium was changed to either low magnesium (0.02 mM added) or normal magnesium (1 mM) with 10% dialyzed serum. The cultures were washed twice with Tris-buffered saline before this medium change. The medium was replenished every 12 hr. ●---●, Low magnesium; ●—●, normal magnesium. (*a*) Cell protein. (*b*) [^{3}H]Leucine incorporation per protein (1-hr labeling period). (*c*) Intracellular magnesium per protein. (*d*) Intracellular potassium per protein. (*e*) [^{3}H]Thymidine incorporation per protein (1-hr labeling period).

with low or normal levels of magnesium. Two days afterwards, a medium change was made. Twelve hours later, some of the cultures previously in low magnesium medium were changed into normal magnesium medium. The changes that followed are shown in Fig. 2. Restoration of magnesium caused cells to resume growth (Fig. 2*a*). Protein synthesis, magnesium, and potassium content increased sharply, all approaching control levels within 1 hr and remaining at the increased levels thereafter (Fig. 2 *b–d*). The first major increase in the rate of thymidine incorporation, however, was not seen until 12 hr, and it only approached the original level of the control at 24 hr (Fig. 2*e*). At the same time, the control level decreased, which could have occurred because of medium depletion or because of the inhibitory effect of prolonged crowding (9).

DISCUSSION

Not much is known about magnesium and protein synthesis rates *in vivo*. In *in vitro* systems, protein synthesis rates have a bell-shaped curve dependence on magnesium when other

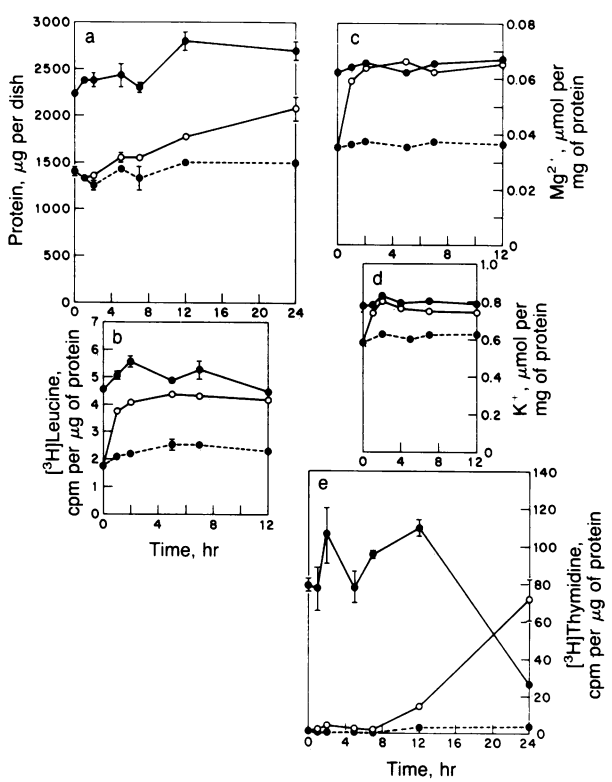

FIG. 2. Effects of restoring magnesium to magnesium-deprived clone 14a cells. Clone 14a cells were plated at 10^5 cells per 60-mm dish in medium containing 10% serum. The medium was replenished at 2 days. At 2.5 days, cells were put into either low magnesium (0.02 mM added) or normal magnesium (1 mM) medium with 10% dialyzed serum. The cultures were washed twice with Tris-buffered saline before this medium change. After an additional 2 days, the medium was replenished. At 2.5 days, cells that had been in low magnesium medium were either switched to fresh normal magnesium medium or were given fresh low magnesium medium. Cells that had been in normal magnesium medium were given fresh normal magnesium medium. The medium was replenished 12 hr later. ●---●, Cells originally in low magnesium medium and kept in low magnesium medium; ○—○, cells originally in low magnesium medium and then changed to normal magnesium medium; ●—●, cells originally in normal magnesium medium and kept in normal magnesium medium. (*a*) Cell protein. (*b*) [^{3}H]Leucine incorporation per protein (30-min labeling period). (*c*) Intracellular magnesium per protein. (*d*) Intracellular potassium per protein. (*e*) [^{3}H]Thymidine incorporation per protein (30-min labeling period, with the data point graphed at the midpoint of the labeling period).

variables are kept constant. However, the optimal magnesium concentration is highly dependent on the concentration of other ions (10) and on the concentration of the various substrates, cofactors, and initiation factors. The concentrations of all these constituents have not been measured simultaneously in cells. Furthermore, the value for the concentration of free intracellular magnesium available for protein synthesis has not been firmly established. Some recent measurements of the free magnesium concentration have ranged from 0.2–0.6 mM by nuclear magnetic resonance methods (11) to 0.8–1.35 mM by electrode measurements, "null-point" determinations with A23187, and nuclear magnetic resonance methods (12).

Previous work suggested that intracellular magnesium could regulate protein synthesis rates (3). In the present experiments, the use of cells in which calcium concentration is left at physiological levels as well as the use of several time points makes the evidence stronger. We have observed that a small change in magnesium at an early time point is associated with a change in protein synthesis rates. This is evidence that intracellular magnesium is not present at an excess concentration, but it is instead present at a regulatory concentration for protein synthesis. At later time points, changes in magnesium concentration and protein synthesis rates are parallel and almost proportional to each other, providing further evidence that the protein synthesis rates are regulated by intracellular magnesium.

In these experiments, protein synthesis rates are observed to be sensitive to small variations in intracellular magnesium. Since variations in intracellular magnesium content are known to occur in "physiological" stimulations of cells (13, 14) and in the inhibition of growth by high cell density (9), it is plausible that magnesium plays a role in the regulation of protein synthesis that occurs at those times.

Intracellular potassium content is also known to change during physiological alterations of growth. However, intracellular potassium can be varied considerably without effect on the growth rate of cells (3, 15). The changes in potassium observed in Figs. 1*e* and 2*e* are therefore unlikely to have direct effects on protein synthesis. Since potassium concentration has been shown to affect the magnesium dependence of *in vitro* protein synthesis (10), the two ions could act together in affecting protein synthesis rates.

It is also likely that magnesium and protons interact with each other in regulating cell growth. Intracellular pH increases when quiescent cells are stimulated by mitogens (16, 17). Magnesium and protons compete with each other for binding to the γ phosphate of ATP. Since the pK value for proton binding to the γ phosphate of ATP is 6.5 (18), changes in pH are likely to affect the binding of magnesium to ATP (19). The decrease in protons that occurs with increased pH should shift $HATP^{3-}$, which is inhibitory for many phosphorylation reactions, to $MgATP^{2-}$, which is the substrate for these reactions (20, 21). Thus, protein synthesis could be affected both directly and indirectly by altered pH through its effect on the distribution of magnesium among adenine nucleotides.

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