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Lack of effect of transmembrane gradient of magnesium and sodium on regulation of cytosolic free magnesium concentration in rat lymphocytes

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Abstract

The regulation of the intracellular concentration of Mg^{2+} ($[Mg^{2+}]i$) is not fully understood. The level of Mg in lymphocytes is a good predictor of total body Mg status. We measured $[Mg^{2+}]i$ and total Mg in rat lymphocytes by using, respectively, the fluorescent Mg^{2+} indicator mag-fura-2 and atomic absorption spectrophotometry. The basal $[Mg^{2+}]i$ in rat lymphocytes was 328 ± 23 μmol/l. An elevation to 5 mmol/l or the removal of extracellular Mg^{2+} did not affect $[Mg^{2+}]i$. A reduction in extracellular Na^+ did not influence $[Mg^{2+}]i$ for 60 min. The total Mg concentration in lymphocytes also remained stable. Results suggest that the permeability of the plasma membrane to Mg^{2+} is very low, and that Na^+/Mg^{2+} exchange is not involved in the regulation of $[Mg^{2+}]i$ in rat lymphocytes. © 1997 Elsevier Science B.V.

Keywords: Cytosolic magnesium; Lymphocyte; Mag-fura-2

1. Introduction

Intracellular free Mg^{2+} is essential to such cell functions as enzyme activity, ion transport, and modulation of membrane receptors [1]. The mechanisms that maintain the $[Mg^{2+}]i$ at an optimal level are not fully understood. The development of fluorescent Mg^{2+} indicator has facilitated the study of such mechanisms [2]. A low permeability of the cell membrane to Mg^{2+} and to the Na^+/Mg^{2+} exchange process is reportedly present in some types of cells [3–5]. The changes in $[Mg^{2+}]i$ by a variety of physiological stimuli have been reported [6–8]. Lymphocytes are commonly used to evaluate the cellular metabolism of cations such as Na^+, H^+, Ca^{2+} and Mg^{2+} in patients with various diseases. The level of Mg in lymphocytes has been shown to be a good predictor of total body Mg status [9,10].

Although several investigators have studied the regulation of Mg^{2+} in lymphocytes, they usually measured either $[Mg^{2+}]i$ or total Mg concentration [9–13], with only a few of them measuring both concentrations [14]. Because a large portion of the intracellular Mg is bound to ATP, $[Mg^{2+}]i$ is influenced by reciprocal changes in intracellular ATP, and

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it is possible that a change in $[Mg^{2+}]i$ does not reflect Mg^{2+} transport across the plasma membrane [15]. We therefore considered it necessary to investigate the relationship between changes in $[Mg^{2+}]i$ and those in total Mg concentration.

The present study assessed the permeability of the plasma membrane to Mg^{2+}, and evaluated the possibility of a Na^+/Mg^{2+} exchange process by measuring the effect of a decrease in the transmembrane Na^+ gradient on $[Mg^{2+}]i$ in rat lymphocytes. We evaluated Mg^{2+} transport across the plasma membrane by measuring both $[Mg^{2+}]i$ and total Mg concentrations.

2. Methods

Male rats of the Wistar strain ($n = 40$), aged 10–11 weeks (Charles River, Japan) were used. After the animals had been anesthetized with intraperitoneal ketamine (15 mg/100 g of body weight), the thoracic cavity was opened to remove the thymus. The thymus was rinsed in RPMI 1640 medium (Gibco) and the attached blood vessels and connective tissue were removed. The thymus was minced and the crude suspension was passed through a nylon mesh and layered on Ficoll-metrizoate with a density of 1087. It was then centrifuged at $800 \times g$ for 20 min. The isolated lymphocytes were washed once in RPMI 1640 medium. The cells were counted and suspended in RPMI 1640 medium at a concentration of 5×10^7 cells/ml. The standard HEPES buffer contained NaCl, 145 mmol/l, KCl, 5 mmol/l, $MgSO_4$, 1 mmol/l, $CaCl_2$, 1 mmol/l, glucose, 5 mmol/l, and HEPES, 10 mmol/l, at pH 7.4.

2.1. Study 1: Effects of extracellular Mg^{2+} concentration on $[Mg^{2+}]i$ and total Mg

To assess whether changes in extracellular Mg^{2+} might affect $[Mg^{2+}]i$ and total Mg in the lymphocytes, the cells were resuspended in various concentrations of Mg^{2+}–HEPES buffer solutions that contained concentrations of $MgSO_4$ that were nominally zero (low-Mg^{2+}), 1 mmol/l (standard), or 5 mmol/l (high-Mg^{2+}). Incubation of the cells in the different Mg^{2+} solutions for 30 to 60 min at room temperature was followed by measurement of $[Mg^{2+}]i$ and total Mg.

2.2. Study 2: Effects of extracellular Na^+ concentration (transmembrane Na^+ gradient) on $[Mg^{2+}]i$ and total Mg

Lymphocytes were divided into two batches. To reduce the transmembrane Na^+ gradient, one batch was resuspended in low-Na^+–HEPES buffer. We reduced the extracellular Na^+ concentration to 37 mmol/l by adding three volumes of Na^+-free-HEPES buffer (in which NaCl had been iso-osmotically replaced by *N*-methyl-D-glucamine) to one volume of standard HEPES buffer. After the lymphocyte suspension had been maintained at room temperature for periods of 30 to 60 min, $[Mg^{2+}]i$ and total Mg were measured.

2.3. Measurement of $[Mg^{2+}]i$

A suspension of lymphocytes that contained approximately 5×10^7 cells/ml was loaded with mag-fura-2/acetoxymethyl ester, 2 μmol/l, (Molecular Probes, Eugene, OR) together with 0.02% Pluronic F-127 (Molecular Probes) for 30 min at 37°C. Extracellular mag-fura-2/acetoxymethyl ester was removed by centrifugation at 2000 g for 5 min at room temperature. The lymphocytes were resuspended at approximately 1×10^7 cells/ml in the HEPES buffer described above. These suspensions were re-incubated for 7 min at 37°C before fluorescence was recorded, to ensure complete de-esterification of the mag-fura-2/acetoxymethyl ester. For measurements of fluorescence, 2.5-ml aliquots of the cell suspension were transferred to a quartz cuvette maintained at 37°C in a spectrofluorometer (SPEX Fluorolog; SPEX Industries, Edison, NJ). Fluorescence signals were monitored at 510 nm with alternate excitation with UV light at 340 and 380 nm. $[Mg^{2+}]i$ was calculated from the ratio of the fluorescence at the two excitation wavelengths according to the formula [2]:

$$[Mg^{2+}]i = K_D(R - R_{min}/R_{max} - R)(S_{f2}/S_{b2})$$

where the K_D (dissociation constant) was 1.5 mmol/l, R was the fluorescence ratio at the excitation wavelengths (340/380 nm) of the samples. R_{max} was the fluorescence ratio at the excitation wavelengths (340/380 nm) under Ca^{2+}-saturated conditions. We

assumed that the fluorescence ratio under Ca^{2+}-saturated conditions was equal to that under Mg^{2+}-saturated conditions. R_{min} was the fluorescence ratio at the excitation wavelengths (340/380 nm) with zero levels of Mg^{2+} and Ca^{2+}. S_{f2} and S_{b2} were the fluorescence intensities at 380 nm for mag-fura-2 with zero Mg^{2+} and excess Mg^{2+}, respectively.

To determine R_{max}, the cells were lysed with digitonin, 50 μmol/l, (Sigma Chemical Co., St. Louis, MO) in the presence of Mg^{2+}, 1 mmol/l, and Ca^{2+}, 1 mmol/l, and R_{min} was then obtained by chelating Mg^{2+} and Ca^{2+} with EDTA, 6 mmol/l, followed by adjustment of pH to 8.3 with Tris, 7 mmol/l. We subtracted from the measured values the autofluorescence from the unloaded lymphocytes, the test agents, and the medium.

Because some leakage of mag-fura-2 occurs from the lymphocytes, it can form complexes with extracellular Mg^{2+} and Ca^{2+} [2]. Therefore, a correction for the extracellular leakage of mag-fura-2 is needed to avoid an overestimation of $[Mg^{2+}]i$ present in the buffer containing Mg^{2+} and Ca^{2+}. A correction was performed using EDTA similar to the correction for extracellular fura-2 using EGTA [16,17]. To calculate the extracellular mag-fura-2, we determined the change in the fluorescence of mag-fura-2 after chelating the extracellular Mg^{2+} with a sufficient amount of EDTA. A rapid drop in the fluorescence signal at 340 nm after the addition of EDTA was considered to reflect the contribution of the extracellular leakage of mag-fura-2 from the cells. We also added EDTA after the lysis of the cells to estimate the total fluorescence associated with mag-fura-2. The ratio of fluorescence change after EDTA in a suspension of intact cells to that in the total dye was regarded as the percentage of the dye that leaked from the cells. To chelate extracellular Mg^{2+}, EDTA, 3 mmol/l, was added to the buffer with nominally zero Mg^{2+}/Ca^{2+}, 1 mmol/l, and Mg^{2+}, 1 mmol/l/Ca^{2+}, 1 mmol/l, and EDTA, 7 mmol/l, to the buffer with Mg^{2+}, 5 mmol/l/Ca^{2+}, 1 mmol/l. Measurements were performed in duplicate. The intra-assay coefficient of variation for $[Mg^{2+}]i$ was 2.2%.

2.4. Measurement of total Mg

The lymphocyte suspension was washed twice in the medium that was nominally free of Mg^{2+} and the supernatant was removed. A volume of 1 ml of HNO_3 was added to the sedimented cells. The mixture was placed in the decomposition vessel (Uniseal Decomposition Vessels, Ltd., Israel), and heated at 140°C for 120 min. Total Mg was measured by atomic absorption spectrophotometry (AA-780, Nippon Jarrell Ash, Tokyo) after wet incineration of samples. The intra-assay coefficient of variation for total Mg was 3.6%.

Table 1
Effects of extracellular Mg^{2+} concentration on $[Mg^{2+}]i$ in rat lymphocytes

Extracellular Mg^{2+} concentration	$[Mg^{2+}]i$ (μmol/l)		
	0 min	30 min	60 min
Low (nominally zero)		307 ± 22	312 ± 26
Basal (1 mmol/l)	328 ± 23	292 ± 28	309 ± 24
High (5 mmol/l)		322 ± 19	335 ± 33

Values are expressed as means ± SEM. $n = 10$ in all experiments.

2.5. Statistical analysis

Values are expressed as means ± SEM. Data were evaluated by analysis of variance for repeated measures. A level of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Study 1: Effects of extracellular Mg^{2+} concentration on $[Mg^{2+}]i$ and total Mg

The basal $[Mg^{2+}]i$ in the lymphocytes was 328 ± 23 μmol/l (Table 1). The $[Mg^{2+}]i$ did not differ between the cells suspended for 60 min in the nomi-

Table 2
Effects of extracellular Mg^{2+} concentration on total Mg concentration in rat lymphocytes

Extracellular Mg^{2+} concentration	Total Mg concentration (nmol/10^6cells)		
	0 min	30 min	60 min
Low (nominally zero)		1.14 ± 0.08	1.03 ± 0.06
Basal (1 mmol/l)	1.06 ± 0.08	1.03 ± 0.08	1.02 ± 0.07
High (5 mmol/l)		1.05 ± 0.07	1.06 ± 0.06

Values are expressed as means ± SEM. $n = 10$ in all experiments.

Table 3
Effects of extracellular Na^+ concentration on $[Mg^{2+}]i$ in rat lymphocytes

Extracellular Na^+ concentration	$[Mg^{2+}]i$ (μmol/l)		
	0 min	30 min	60 min
Low (37 mmol/l)		343 ± 42	308 ± 41
Basal (145 mmol/l)	328 ± 37	347 ± 39	337 ± 40

Values are expressed as means ± SEM. $n = 10$ in all experiments.

nally Mg^{2+}-free medium, in a medium with an increase in external Mg^{2+} to 5 mmol/l, or in the standard buffers. It was difficult to detect any change in $[Mg^{2+}]i$ when the external concentration of Mg^{2+} ranged between nominally zero and 5 mmol/l.

The total Mg in the lymphocytes was 1.06 ± 0.08 nmol/10^6cells. No significant change in total Mg was observed when the lymphocytes were incubated for 60 min at room temperature in the different Mg^{2+} solutions (Table 2).

3.2. Study 2: Effects of extracellular Na^+ concentration (transmembrane Na^+ gradient) on $[Mg^{2+}]i$ and total Mg

The partial replacement of extracellular Na^+ with *N*-methyl-D-glucamine affected the ratio of mag-fura-2 fluorescence at excitation wavelengths of 340 and 380 nm. Thus, correction for the leakage of mag-fura-2 from the lymphocytes was required to avoid the influence of *N*-methyl-D-glucamine on the measurement for $[Mg^{2+}]i$. We evaluated the fluorescent signal of extracellular mag-fura-2 in each buffer and subtracted this value from the original signal, thus obtaining an accurate comparison of the lymphocytes suspended in the *N*-methyl-D-glucamine buffer with those suspended in the standard buffer [18].

Reduction of the extracellular concentration of Na^+ to 37 mmol/l produced no significant change in $[Mg^{2+}]i$ within 60 min (Table 3), nor did it significantly affect total concentration of Mg (Table 4).

Table 4
Effects of extracellular Na^+ concentration on total Mg concentration in rat lymphocytes

Extracellular Na^+ concentration	Total Mg concentration (nmol/10^6cells)		
	0 min	30 min	60 min
Low (37 mmol/l)		1.03 ± 0.07	1.04 ± 0.06
Basal (145 mmol/l)	1.02 ± 0.09	1.03 ± 0.09	1.01 ± 0.09

Values are expressed as means ± SEM. $n = 10$ in all experiments.

4. Discussion

Although measurement of the serum Mg level is the usual method for detecting an abnormality in Mg^{2+} homeostasis, a dissociation between the serum and intracellular levels of Mg can be present. Thus, the clinical signs and symptoms of Mg deficiency may be present, despite a normal serum level of Mg [19]. However, it is difficult to measure the levels of Mg in tissues such as cardiac muscle or vascular smooth muscle. Blood cells such as the erythrocytes and lymphocytes were therefore used to evaluate the homeostasis of intracellular Mg^{2+} in several clinical studies [19,20].

In the present experiments, basal $[Mg^{2+}]i$ in lymphocytes of rats was 328 ± 23 μmol/l, within the range of concentration of free Mg^{2+} found in most cells [4,7]. Because those values are lower than it would be predicted from the electrochemical equilibrium, Mg^{2+} is not passively distributed [3]. How this low level of $[Mg^{2+}]i$ is maintained has not been fully established.

In the present experiments, neither the elevation of extracellular Mg^{2+} to 5 mmol/l nor its removal had a significant effect on $[Mg^{2+}]i$ in a 60 min period, suggesting that the permeability of the plasma membrane to Mg^{2+} is very low in lymphocytes during unstimulated conditions. These observations are consistent with results obtained in other tissues [4,15].

In some kinds of cells extracellular Na^+ has been demonstrated to be important to the efflux of Mg^{2+}, and Na^+/Mg^{2+} exchange may be present [4,5]. Gunther and Vormann reported the existence of a Na^+-dependent Mg^{2+} efflux in Mg^{2+}-loaded lymphocytes that had been prepared by incubation with the cation ionophore A23187 [11,12]. However, the present study showed that a reduction in extracellular Na^+ did not influence $[Mg^{2+}]i$. Our results suggest that, although an Na^+-dependent Mg^{2+} efflux may occur in the presence of the extremely high intracellular concentrations of Mg induced by Mg^{2+}-loading, Na^+/Mg^{2+} exchange was not involved in the transport of Mg^{2+} by the plasma membrane under physio-

logical conditions. Evidence for Na^+/Mg^{2+} exchange has been demonstrated in the erythrocytes [3]. Yoshimura et al. [4] reported that Na^+/Mg^{2+} exchange likely regulates $[Mg^{2+}]i$ in platelets. Thus, the results of the present study suggest that lymphocytes differ from erythrocytes and platelets in the mechanisms that maintain a low level of Mg^{2+}. Ryan et al. [9] reported that the level of Mg in the lymphocytes was a more accurate reflection of its levels in skeletal and cardiac muscle than was its level in the erythrocytes. A difference in the effect of Na^+/Mg^{2+} exchange on the mechanisms of Mg^{2+} transport may be responsible for their observations.

Intracellular Mg exists in three different states: bound to protein, complexed to anions, and ionized. A large portion is involved with ATP, ADP, and AMP, with less than 10% of intracellular Mg being in the free ionized form [3]. Because $[Mg^{2+}]i$ is influenced by reciprocal changes in intracellular ATP, it is possible that a change in $[Mg^{2+}]i$ does not always reflect Mg^{2+} transport across the plasma membrane [15]. In addition one could not exclude the possibility that mag-fura-2 inhibited the influx of Mg^{2+} through a channel or exchanger. We confirmed that a change in extracellular Mg^{2+} or a reduction of extracellular Na^+ did not affect the intracellular total Mg concentration. No change of intracellular total Mg concentration showed that neither extracellular Mg^{2+} nor Na^+ significantly affected Mg^{2+} transport across the plasma membrane. Measurement of both the intracellular total Mg concentration and $[Mg^{2+}]i$ could show that an increase or decrease in extracellular Mg^{2+} and a decrease in extracellular Na^+ did not affect the ratio of free Mg to total Mg concentration, and that there was no discrepancy between $[Mg^{2+}]i$ and total Mg concentration.

In conclusion, the present study showed that the permeability to Mg^{2+} was very low and that Na^+/Mg^{2+} exchange was not involved in maintaining $[Mg^{2+}]i$ below the level of electrochemical equilibrium in rat lymphocytes under physiologic conditions. Further research is required to explain in detail the regulation of Mg^{2+}.

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