Blocking the rise of intracellular calcium inhibits the growth of cells cultured in different concentrations of magnesium

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Abstract. Divalent cations, especially calcium and magnesium, have been shown to play an important regulatory role in endothelial and immune cells. To learn more about the interaction of these two metals in the regulation of cell growth, we altered the calcium/magnesium ratio by culturing human endothelial cells, macrophages, and T lymphocytes in media containing different concentrations of magnesium. We observed that the growth of the three cell types was retarded in low extracellular magnesium, and this retardation is particularly evident in highly proliferating cells. High concentrations of magnesium does not exert any effect on cell growth. When (i) calcium influx was blocked by adding the calcium antagonist verapamil, and (ii) calcium release from intracellular stores was inhibited by exposure to TMB-8, the growth of endothelial cells, macrophages, and T lymphocytes was inhibited. In particular, the release of calcium from intracellular stores seems to be more important than its influx in sustaining cell proliferation. Our results indicate that calcium plays a crucial role in mediating cell proliferation independently from the extracellular concentrations of magnesium.

Key words: magnesium, calcium, cell growth, endothelial cell, macrophage, lymphocyte

Calcium is a versatile, intracellular second messenger that regulates many cellular processes including cell proliferation, death and metabolism in different cell types. Indeed, calcium regulates a plethora of intracellular enzymes located in the cytoplasm, nucleus and organelles [1]. In resting cells, the cytosolic free calcium concentration is maintained at a low level (50-150 nM). However, agonists including hormones, growth factors and antigens induce rises in intracellular calcium, via mobilization of calcium from intracellular stores and the extracellular fluid. In particular, the endoplasmic reticulum serves as a rapidly exchanging calcium store, and contributes to the cytosolic calcium-signalling cascade by releasing calcium, mainly through ryanodine (RyR) and inositol triphosphate (IP3R) receptors [2], while the influx of calcium from extracellular fluids is required for a sustained calcium increase and for the full activation of calcium-dependent processes. In particular, recent pharmacological and molecular genetic approaches have revealed the existence
of functional L-type calcium channels (LTCCs) in a variety of non-excitable cells [3]. Indeed, LTCCs have a significant role in calcium influx-mediating T lymphocyte activity and proliferation in vivo and in vitro, and verapamil, an L-type calcium channel blocker, impairs lymphocyte functions [4, 5]. Also, in macrophages and in endothelial cells, the presence of functional LTCCs has been reported and, accordingly, inhibitors of these channels have profound effects on these cells [6, 7].

Magnesium (Mg), the most abundant divalent cation in mammalian cells, is a pivotal cofactor for ATP, polyphosphates such as DNA and RNA, and metabolic enzymes. Mg is thus involved in the regulation of essential cell functions such as metabolism, proliferation, and death [8]. Increases in intracellular Mg levels are reported following cell exposure to growth factors, as well as in lymphocyte activation. Recently, mutations in the Mg transporter gene MAGT1, has been identified in an X-linked, human T-cell immunodeficiency, revealing a role for Mg as an intracellular second messenger, coupling cell-surface receptor activation to intracellular effectors [9]. These novel results suggest the need to explore further the intracellular role of Mg.

Interestingly, Mg has been considered to be a natural calcium antagonist [10], and this is explained, in part, by the evidence that these two alkaline earth metals share the same transporters in most cell types. Very little is known at the cellular level about the effects of an imbalance between Ca and Mg. Therefore we investigated the effects of the L-type calcium channel-blocker verapamil, and TMB-8, which inhibits Ca-induced Ca release to the cytosol [11], on three types of cells, i.e. primary human endothelial cells derived from the umbilical vein (HUVEC), J774.E macrophages, and D10.G4.1 T lymphocytes, all cultured in normal, low or high Mg concentrations to generate an altered Ca:Mg ratio. These cell types have been chosen as endothelial cells, macrophages, and lymphocytes are crucial players in inflammation and immune responses, processes in which Mg is an important participant [12].

Materials and methods

Cell culture

A mouse lymphocyte-like cell line D10.G4.1 (ATCC, Rockville, Maryland, USA) was grown in suspension in RPMI-1640 medium (Sigma Aldrich, Poznan, Poland) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine (Sigma Aldrich), 10% T-STIM (Becton Dickinson), 10 μg/mL mouse IL-1α (eBioscience, Vienna, Austria), 0.05 mM 2-mercaptoethanol (Sigma Aldrich), 40U/mL penicillin G (Sigma Aldrich), and 100 μg/mL streptomycin (Sigma Aldrich).

Macrophage-like J774.E cells were cultured in RPMI-1640 medium (Sigma Aldrich) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 4 mM L-glutamine (Sigma Aldrich) and 50 μg/mL gentamicin (Sigma Aldrich).

After isolation from the umbilical vein, HUVEC were grown in M199 containing 10% FBS, 1 mM glutamine, 1 mM penicillin and streptomycin, endothelial cell growth factor (150 μg/mL), 1 mM sodium pyruvate and heparin (5 units/mL) on 2% gelatin-coated dishes.

A magnesium-free medium was purchased from Invitrogen (San Giuliano M.se, Italy) and used with MgSO₄ to provide a variety of magnesium concentrations. On the basis of previous reports [13, 14], we cultured the cells in medium containing 0.1, 1.0 or 5.0 mM Mg. To inhibit increases of intracellular Ca, we used verapamil and TMB-8 because these compounds have similar molar mass and they are both soluble in water.

Cell proliferation

We first performed experiments to identify the IC50 of verapamil and TMB-8 in HUVEC. Table 1 summarizes the results obtained.

Proliferation assays were performed on HUVEC at low density (7,500/cm²), D10.G4.1 (60,000/mL)

Table 1. The inhibitory concentration (IC₅₀) values for the Ca inhibitors were determined in HUVEC, D10.G4.1, and J774.E cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Verapamil (μM)</th>
<th>TMB-8 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC</td>
<td>47.2±8.5</td>
<td>16.1±3.9</td>
</tr>
<tr>
<td>D10.G4.1</td>
<td>58.2±7.5</td>
<td>16.1±3.9</td>
</tr>
<tr>
<td>J774.E</td>
<td>48.3±7.6</td>
<td>32.6±5.4</td>
</tr>
</tbody>
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The results are means ±SD. Each experiment was repeated at least three times. At 0 time-point, cells were seeded in complete medium and then treated with different concentrations of the two compounds for 48 h.
and J774.E (40,000/mL) cells cultured in medium containing different concentrations of Mg in the presence or absence of verapamil and TMB-8 (both 25 μM), alone or in combination. Four hours before the end of each time-point, the medium was replaced with fresh medium containing 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide (MTT) (Sigma-Aldrich). Then, 100 μL of lysis buffer were added to each well and the plates were incubated for an additional four hours at 37°C. Absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm. Calibration curves of 20,000-400,000 cells/mL served as standards. A commonly used formula to report the percentage of suppression in such studies was adapted from Brusko et al. [15].

**Measurement of intracellular free Ca with Fura-2/AM**

HUVEC were seeded onto coverslips or in 24-well plates. The cells were incubated in 0.1, 1.0 or 5.0 mM Mg for 24 h. Then Fura-2/AM (10 μM) was added to the culture medium for 60 min. Labeled cells attached to coverslips were washed and studied by fluorescence microscopy. The cells in the 24-well plates were washed with a buffer containing NaCl 125 mM, KCl 5 mM, MgSO₄ 1.2 mM, CaCl₂ 2 mM, glucose 6 mM, Hepes-NaOH buffer 25 mM (pH 7.4), removed by trypsinization, suspended in the aforementioned buffer. They were then analyzed using a spectrophotometer with excitation wavelengths of 360 nm and emission at 450 nm [16]. Results were normalized in relation to 0.1 mM Mg.

![Figure 1. Intracellular Ca in HUVEC cultured in 0.1, 1.0 or 5.0 mM Mg.](image)

HUVEC were seeded on coverslips. After 24 h culture in 0.1, 1.0 or 5.0 mM Mg, the cells were loaded with Fura-2/AM for 1 h and (A) photographed or (B) intracellular Ca was measured as described in the “Materials and methods” section. Values are means ± SD from four independent experiments performed in triplicate.
to the baseline fluorescence of the cells and data were expressed as a percentage increase in relation to the baseline.

**Statistical analysis**

For intracellular Ca measurements, statistical significance was determined using Student’s *t* test and *p* values set at less than 0.05.

For proliferation assays, statistical analysis was performed using Statistica® (version 6.0; Statistica Software). Results were expressed as mean ± standard deviation of at least three separate experiments. Statistical multiple comparison was estimated using the Kruskal-Wallis ANOVA, and the comparison between two groups was estimated using the Mann-Whitney U test. In all cases, *p*-values lower than 0.05 were considered statistically significant.

**Results**

**Intracellular levels of Ca in HUVEC cultured in 0.1, 1.0 and 5.0 mM Mg.**

The growth of HUVEC was inhibited when they were cultured in low extracellular Mg. Since (i) Ca is essential for cell proliferation and (ii) Ca and Mg compete for the same transporters, we evaluated whether differences in intracellular Ca could be detected in HUVEC cultured in 0.1, 1.0 or 5.0 mM Mg. While 1.0 mM is considered to be the physiological concentration of the cation, 0.1 mM Mg, which is detected in rodents after eight days of an experimental diet, has been widely used in various cultured mammalian cells to study the effects of low Mg [8], and 5 mM is a very high concentration previously used in *in vitro* studies [13]. Culture in 0.1 mM Mg generates an increase in the Ca:Mg ratio (Ca:Mg ratio 18), while culture in 5.0 mM Mg decreases this ratio. The cells were cultured in different concentrations of Mg for 24 h, and loaded with Fura-2 for 1 h. Fura-2/AM imaging microscopy was used to study intracellular Ca distribution (figure 1A). No major differences were observed under the experimental conditions tested. The cells showed a uniform, diffuse stain. When the Ca content was measured, we observed a decrease in intracellular Ca in 0.1 and 5.0 mM Mg versus the controls in 1.0 mM Mg (figure 1B). Interestingly, no significant variations in intracellular Mg levels were detected in HUVEC at various time-points between 30 min and 24 h (not shown).

![Figure 2. Intracellular Ca in HUVEC exposed to verapamil and TMB-8.](image)

After 24 h culture in the presence of verapamil (25 μM) or TMB-8 (25 μM), the cells were loaded with Fura-2/AM and intracellular Ca was measured as described in the “Materials and methods” section. Values are means ± SD from four independent experiments performed in triplicate.
Verapamil and TMB-8 effects on HUVEC proliferation

To understand the role of intracellular Ca in modulating the growth of HUVEC cultured in different concentrations of extracellular Mg, we used verapamil, a commonly employed Ca blocker, and TMB-8, an inhibitor of Ca-induced Ca release into the cytosol [11]. We evaluated intracellular Ca in HUVEC exposed for 24 h to verapamil and TMB-8 (both at 25 μM). Verapamil and TMB-8 slightly, but reproducibly, decreased intracellular Ca (figure 2).

Verapamil and TMB-8 were added alone or in combination to HUVEC cultured in different concentrations of extracellular Mg for 24, 48 and 72 h. Figure 3 shows that culture in 0.1 mM Mg inhibited HUVEC proliferation (about 15% versus control after 72 h), while 5.0 mM had no significant effects. Verapamil and TMB-8 inhibited the proliferation of the cells in 0.1, 1.0 and 5.0 mM Mg (35% and 80% after 72 h, respectively). When the two compounds were added together, the inhibition of cell growth was particularly evident (40% after 24 h, 90% after 72 h).

Verapamil and TMB-8 effects on the proliferation of D10.G4.1 and J774.E cells cultured in 0.1, 1.0 or 5.0 mM Mg

The mouse lymphocyte-like cell line D10.G4.1 and macrophage-like J774.E cells were grown in 0.1, 1.0 and 5.0 mM Mg, with or without verapamil and TMB-8. Figures 4-5 show that 0.1 mM Mg inhibited the proliferation of D10.G4.1 and J774.E...
Magnesium/Calcium and cell growth

Figure 4. The effects of different concentrations of Mg, with or without verapamil and TMB-8, on the proliferation of D10.G4.1 cells.

Cells were cultured in 0.1, 1.0 or 5.0 mM Mg without (CONTROL) or with verapamil (VER) or TMB-8 for 24, 48, and 72 h. The values are means ±SD from at least three independent experiments. Different letters indicate significant effect (p < 0.05) of treatments within the same time-period studied: (a, b, c) effect of various Mg concentrations; (w, x, y, z) effect of Ca blockers on cells cultured in the control Mg concentration (1 mM).

Discussion

The results shown here confirm that Mg is implicated in altering cell proliferation. Indeed, low extracellular Mg retards the proliferation of HUVEC, J774.E macrophages, and D10.G4.1 T lymphocytes. It is noteworthy that the inhibition of cell growth by low Mg seems to be more evident in highly proliferating cells, i.e. J774.E cells, as compared to HUVEC whose doubling time is approximately twice as long. High concentrations of extracellular Mg exert no significant effect on cell proliferation. Interestingly, extracellular Mg concentrations do not alter intracellular Mg levels in HUVEC (data not shown). We have not determined intracellular Mg levels in J774.E macrophages or D10.G4.1 T lymphocytes, but data from the literature and from our studies on HUVEC show that, in general, total intracellular Mg is not significantly influenced by extracellular Mg concentrations. It would be interesting to know whether the intracellular Mg concentrations...
distribution of the cation is altered, but very little information is available on this issue currently because of the technical difficulties involved in tracing intracellular Mg. Only very recently, a complex and novel technical approach for studying intracellular Mg distribution has been described [17].

Although the inhibitory effect of low extracellular Mg has been described in various cell types [8, 14, 18-21], the mechanisms involved are not well understood. We anticipate that an imbalance within the Ca/Mg ratio might play a role.

Using HUVEC, we first evaluated the impact of different concentrations of Mg on intracellular Ca. We found a significant decrease in intracellular Ca, both in cells in 0.1 and in 5.0 mM Mg, when compared to controls. While the result obtained in HUVEC cultured in high extracellular Mg can be explained by the well-known antagonism between Mg and Ca [10], the decrease in intracellular Ca in Mg-deficient cells is rather puzzling. Since Mg is essential for ATP to become biologically active, we hypothesize that a decrease in ATP activity might impair the balance of ions through the membranes. Alternatively, Mg deficiency might directly alter the function of Ca transporters or Ca sensor receptors. We then investigated the effects of a commonly used Ca blocker, verapamil, and an inhibitor of Ca-induced Ca release into the cytosol [11], TMB-8, on HUVEC proliferation upon exposure to various concentrations of Mg. Under physiological conditions, i.e. culture medium containing 1.0 mM Mg, verapamil and TMB-8 exert an inhibitory effect on cell proliferation, thus mimicking what happens upon culture in low extracellular Mg. In particular, these two compounds exacerbate the inhibitory effect of Mg deficiency. The fact that verapamil and TMB-8 also inhibit

**Figure 5.** The effects of different concentrations of Mg, with or without verapamil and TMB-8, on the proliferation of primary J774.E. Cells were cultured in 0.1, 1.0 or 5.0 mM Mg without (CONTROL) or with verapamil (VER) or TMB-8 for 24, 48, and 72 h. The values are means ± SD from at least three independent experiments. Different letters indicate significant effect (p < 0.05) of treatments within the same time-period studied: (a, b, c) effect of various Mg concentrations; (w, x, y, z) effect of Ca blockers on cells cultured in the control Mg concentration (1 mM).
endothelial growth in 1.0 and 5.0 mM Mg, suggests the crucial and direct involvement of Ca in driving the events leading to endothelial cell division.

Similarly, the growth of J774.E macrophages and D10.G4.1 T lymphocytes was also inhibited by verapamil and TMB-8 when added not only to medium containing physiological concentrations, but also medium containing high concentrations of Mg.

In the three cell types used in this study, blocking Ca release from intracellular stores seemed to be more important than Ca influx in inhibiting cell proliferation. This result could be explained by the fact that in non-excitable cells, Ca release from intracellular stores triggers Ca influx from the extracellular space [3]. Accordingly, a synergistic effect is observed when verapamil and TMB-8 are added together.

Since Mg has been proposed as a natural Ca antagonist and has been shown to be a potent L-type calcium channel inhibitor [22, 23], it is interesting to note that high extracellular Mg does not seem to mimic the action verapamil on the proliferation of the cells used in this study. Indeed, while we observed no alteration of cell growth in cells in 5.0 versus cells in 1.0 mM Mg, high extracellular Mg did not prevent the inhibitory effects of verapamil and TMB-8.

More studies are needed to focus on the contribution of endoplasmic reticulum-Ca release through ryanodine receptors and inositol trisphosphate receptors (IP3Rs) in our cell systems.

**Limitations of the study**

A limit of our study is the lack of data on the intracellular Ca and Mg concentrations after culture in the different concentrations of extracellular Mg, with or without verapamil or TMB-8.

**Conclusions**

Our principal conclusions are as follows:

- culture of HUVEC in 0.1 or 5.0 mM Mg decreases intracellular calcium levels;
- different cell types respond to various concentrations of extracellular Mg in a similar manner. Indeed, low extracellular Mg inhibits proliferation, while high Mg exerts no effect in HUVEC, J774.E and D10.G4.1 cells. In particular, we point to the fact that the faster the cells grow, the more sensitive they become to low extracellular Mg concentrations; blocking the increase in intracellular calcium inhibits cell proliferation independently from the extracellular concentrations of Mg, suggesting a crucial role for Ca in mediating cell proliferation.

**Disclosure**

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**References**


