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# REGULATION OF MAGNESIUM CONTENT DURING PROLIFERATION OF MAMMARY EPITHELIAL CELLS (HC-11)

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## 1. ABSTRACT

To study the role of Magnesium in the regulation of cell proliferation we characterized the proliferation behaviour of HC-11 mammary epithelial cells that were grown in media containing low to high Mg concentrations. Cells grown under control conditions (0.5 mM Mg in the medium) or in the presence of high (H) Mg (45 mM) displayed similar log-phases and reached confluence in 72h. In the presence of low (L) Mg (0.025 mM) the cells exhibited a reduced growth rate and did not reach confluence at 72h. Intra cellular total Mg increased from 12 to 36h of culture in all cells examined but returned to basal levels in those cells which reached confluence (i.e., control and H-Mg cells). Intra cellular Mg increased independent of mitosis-induced changes of volume and adenine nucleotides pools but correlated with an increased percentage of cells in the S phase and with total nucleic acid contents. These bell-shaped changes of intra cellular Mg were less evident in L-Mg cells, likely due to a combination of low Mg levels in the medium and decreased growth rate. Changes in membrane potential and pH were important factors that contributed to maintaining intra cellular Mg at physiologic levels in the face of increased or decreased availability of extra cellular Mg. H-Mg cells were depolarised and more acidic than control cells; conversely, L-Mg cells showed a pattern of hyperpolarization and alkalinization. These results lend support to the concept that Mg may be involved in regulating cell proliferation, and show that cells maintain adequate levels of intra cellular Mg, and hence their proliferation potential, even under conditions of extreme changes of extra cellular Mg.

## 2. INTRODUCTION

Several lines of evidence indicate that a correlation exists between magnesium [1](Mg) availability and cell proliferation (reviewed in 1). Extra cellular Mg stimulates proliferation of yeast cells, epidermal and endothelial cells, and several other cell types (2-4). Moreover, $[Mg]_i$ increases in MDCK cells exposed to mitogenic stimuli (5, 6). It was also shown that, in general, tumor cells contain more Mg than normal cells and may grow in the presence of lower concentrations of extra cellular Mg compared to their normal counterparts (7, 8).

From a biochemical viewpoint Mg plays an important role in reactions that are crucial to cell proliferation like e.g., DNA duplication, protein synthesis, energy metabolism and transphosphorylations (9). Intra cellular Mg consists of bound and free ionized Mg, referred to as $[Mg]_I$. Intra cellular Mg, and its distribution in free or bound pools, is influenced by factors like *i)* electrochemical gradient across plasma membrane; *ii)* concentration and types of binding species (proteins, phospholipids and other phosphate-containing compounds, nucleic acids, poliamines, etc.); *iii)* specific membrane-associated transport mechanisms. Adenine nucleotides are the major Mg-binding species, as $[Mg]_i$ increases proportionally to ATP hydrolysis. Indeed, the binding between Mg and phosphate can be used to evaluate $[Mg]_i$ by $^{31}$P-NMR (10, 11). As it regards the importance of chemical *vs* electrochemical-driven gradients it is worth noting that $[Mg]_i$ roughly corresponds to $[Mg]_o$ (0.2-0.8 mM *vs* 0.4-0.5 mM, respectively); therefore, electrochemical gradients (generated by membrane potential and pH) are more important than chemical gradients in determining Mg movements across the plasma membrane (12). Other mechanisms are nonetheless involved in regulating intra cellular Mg, as electrochemical forces per se would generate levels of Mg one order of magnitude higher than

those determined under physiologic conditions. These mechanisms have been identified in active transport mechanisms (ATPases, ion exchangers) (13) which have been cloned and characterized in detail in bacteria and yeast (14, 15). Unfortunately, these transport mechanisms have not been characterized at the molecular level in eukaryotic cells.

From a pathophysiologic viewpoint, magnesium deficiency associates with hypertension and cardiovascular diseases or diabetes (16, 17), and in some cases Mg supplementation has proven to be beneficial (18). In considering the biological effects of Mg availability and their possible consequences on disease states one should distinguish acute and chronic Mg changes. Acute modifications of Mg availability influence primarily the proliferation rate and/or survival of normal cells. Chronic modifications, probably more frequent and of greater pathophysiologic relevance, result in a redistribution of intra cellular Mg in compartmentalised *vs* cytoplasmic pools or in bound *vs* free pools; this eventually serves a mechanism to compensate for and adapt to increased or decreased Mg availability (1).

In a previous work we described the effects of an acute extra cellular Mg deficiency on the proliferation of normal and tumour mammary epithelial cells. We showed that Mg deficiency decreased and eventually halted the growth of normal immortalized HC-11 cells but not of their tumour counterparts MCF-7 cells; we also demonstrated that Mg influenced cell proliferation by modulating the expression of the cell cycle inhibitor protein p27 (19). The present study was aimed at obtaining an-in-depth characterization of how intra cellular Mg changed in relation to cell proliferation, an information which may be of value to further establish cause-and-effect relations between Mg availability and cell growth. For this purpose we used HC-11 cells grown in the presence of normal Mg, and clones which were chronically adapted to grow in high or low Mg (20). The Mg content of these cells was determined in relation to cell cycle distribution and volume changes, electrochemical forces determining Mg influx, and intra cellular Mg binding factors like adenine nucleotide pools.

## 3. MATERIALS AND METHODS

### 3.1. Cell cultures

Human mammary epithelial cells (HC-11) were seeded at 6000 cells/cm$^2$ in RPMI with 10% FCS, using cells collected from confluent cultures, namely grown for about 84h.

H-Mg and L-Mg cells were selected in our laboratory as described by Sgambato *et al.* (20) and maintained in 45 mM or 0.025 mM Mg-containing RPMI. L-Mg cells were grown in Mg-free RPMI (Invitrogen) with Mg-free FCS, obtained by dialysis as previously described (21). Serum physiologic Ca concentration was obtained by re-addition of 1.3 mM $CaCl_2$ to dialyzed serum. Unspecific effects due to dialyzed serum were checked by comparing growth and biological behaviour of HC-11 cells grown in regular RPMI + FCS medium with those grown in Mg-free + FCS which was reconstituted by adding the physiologic concentrations of Mg (20). For H-Mg cells the medium was supplemented with the desired Mg concentration (as $MgSO_4$). The presence of excess Mg in the culture medium increased osmolarity by 90 mOs. This relatively mild hyper-osmolarity (20%) was not corrected by reducing Na concentration as $[Na]_o$ served a driving force for the plasma membrane Na-Mg exchange, one of the best established counter-transport mechanisms responsible for Mg efflux from eukaryotic cells (22). Doubling time of cell cultures was calculated at the exponential phase of growth, as described in (20). Cell volume was derived from cell diameter (cytofluorimetric measurements) of the three cell populations, assuming that cells were spherical. Cell Mg concentrations were extrapolated from cell volume, considering that most of the volume was accounted for by water.

### 3.2. Magnesium and nucleic acids assays

Cell total Mg was assayed on acidic extracts of cells. In order to remove extra cellular Mg contamination, culture plates were rapidly washed twice with cold PBS which contained 0.5 mM EDTA in the first wash. Ion extraction was performed by adding 3 ml of $HNO_3$ 1.0 N to plates, left overnight on a shaking surface. The next day acidic extract were carefully collected and centrifuged to remove cell debris. Intra cellular Mg content was measured on supernatants by atomic absorption spectrophotometer (Perkin-Elmer). Parallel plates were used to perform cell counts and protein evaluation. Mg content of H-Mg and L-Mg media was routinely checked by AAS.

Nucleic acids were measured spectro-photometrically on phenol/chloroform extracts of 1x10$^6$ cell, assuming $OD_{260nm}$ = 1 for 50 microg/ml of double-stranded DNA. Purity was judged based upon $OD_{260nm}$ /$OD_{280nm}$ ~ 1.8.

### 3.3. Adenine nucleotides assays

Adenine nucleotides were assayed on cell acidic extracts by HPLC, as described in (23) with minor modifications (24). Proteins were evaluated by Biorad microassay.

### 3.4. Cytofluorimetric analyses

Cell diameter, cell cycle distribution, membrane potential, and intracellular pH were evaluated by cytofluorimetric analyses on a Coulter Epics 753 (Coulter). Samples of approximately 1x10$^6$ trypsinized and PBS washed cells were used for each evaluation. Cell diameter was assessed by comparing the florescence signals of our samples to that of standard 8 micron, 10 micron and 20 micron beads (Coulter). Cell cycle distribution was assayed on fixed and propidium iodide-stained cell samples as previously described (16). Membrane potential was evaluated on fresh cells loaded with 400 nanoM of the fluorescent indicator DiSBA-$C_4$-(3) (Molecular Probes), following the protocol described in (25). Intracellular pH was evaluated on fresh cells loaded with 10 microM BCECF-AM (Sigma), as described (26).

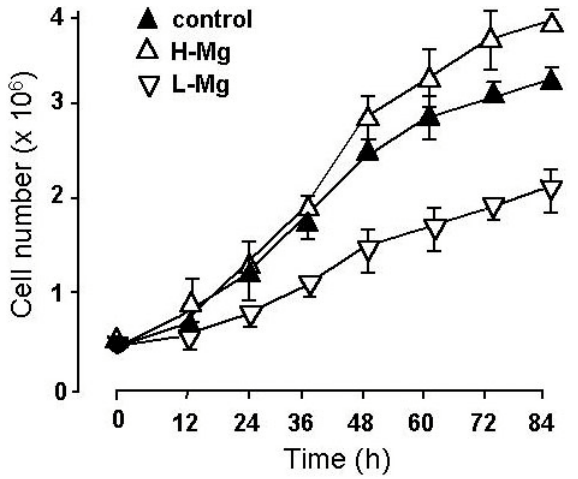


**Figure 1.** Growth rate of HC-11 cells in control, high and low magnesium media. Cell cultures were seeded at a density 6000 cells/cm$^2$, from confluent cultures, and cell counts performed at the indicated time intervals. In H-Mg and L-Mg cultures cells were adapted to grow in 45.0 mM and 0.025 mM Mg-containing media, respectively, as described in Materials and Methods. Values represent means +/- SEM of 5 separate cultures.

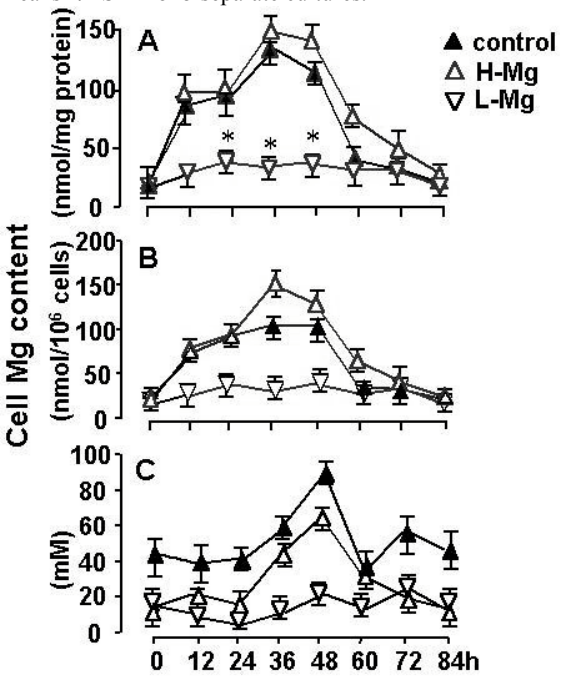


**Figure 2.** Cell total Magnesium content of HC-11 cells during growth in control, high and low magnesium media. Cell cultures were seeded at a density 6000 cells/cm$^2$, and total magnesium was measured in acidic extracts by atomic absorption spectrometry, as described in Materials and Methods. In panel A, Mg is normalized to cell proteins; in panel B, to cell number, and in panel C, to cell volume. Cell volumes were extrapolated from flow cytometric determinations of cell diameters as described in Materials and Methods. The values at time 0 correspond to that of 84h cultures, namely to that of cells that were seeded at time 0. Data are mean +/- SEM of 5 separate experiments, **P* less than 0.05 vs corresponding value at time 0.

### 3.5. Statistical analysis

Data are given as means +/- SEM of at least three separate experiments. In the figures, SEM are indicated by vertical bars; values without vertical bars have SEM within the symbols. Statistical analyses were performed by unpaired Student's *t* test, and differences were considered significant when *P* less than 0.05. Other details are given in the legends to Figures and Tables.

## 4. RESULTS AND DISCUSSION

### 4.1. Cell Magnesium content during proliferation

Figure 1 reports the growth rate of HC-11 human mammary epithelial cells grown for 84 h under control or high (H) or low (L) Mg conditions. The growth rate of control and H-Mg cells was exponential from 24h to 48h; after 60h cells approached microscopically evident confluence and the growth rate decreased. Compared to this pattern the growth rate of L-Mg cells was lower from 12h to 48h and decreased further after 60h, although these cells did not reach confluence within the experiment time. Cell growth was evaluated also by calculating the doubling time (DT) during exponential growth. The DT of H-Mg cells was 25h, about 10% shorter than that of control cells (28h), but increased almost twofold in the case of L-Mg cells (43h).

The different growth rates of control and H-Mg or L-Mg cells was exploited to characterize the regulation of intra cellular Mg in cells which proliferated in the presence of varying amounts of extra cellular Mg. As shown in Figure 2A, total Mg content of control and H-Mg cells increased with time, reaching a maximum ~7 fold increase at 36h of culture, but decreased after 48h and returned to initial values at 84h, that is when both cell populations reached confluence. L-Mg cells, although grown in 20-fold lower extra cellular Mg compared to control cells, still exhibited a bell-shaped changes of total Mg content, reaching a statistically significant (*P* less than 0.05) twofold increase over baseline values at 24h. Moreover, the Mg content of L-Mg cells remained above initial values also after 48h of culture, a finding consistent with sustained growth rate and delayed cell confluence. In Figure 2A cell Mg content was normalized to mg protein, but an essentially similar trend toward a bell-shaped increase and decrease of Mg content was observed also when data were normalized to cell number (Figure 2B) or volume (Figure 2C). Collectively, these experiments showed that total intra cellular Mg increased during cell proliferation but decreased when proliferation ceased and cells reached confluence. These results also showed that proliferation-dependent changes of intracellular Mg were scarcely influenced by extra cellular Mg; in fact (i) H-Mg cells exhibited essentially the same growth rate and 7-fold increase of intra cellular Mg/mg protein as those of control cells in face of a 90-fold increased availability of extra cellular Mg; (ii) L-Mg cells exhibited 50% the growth rate of control cells and a 2-fold increase of intra cellular Mg/mg protein in face of a 20-fold reduced availability of extra cellular Mg.

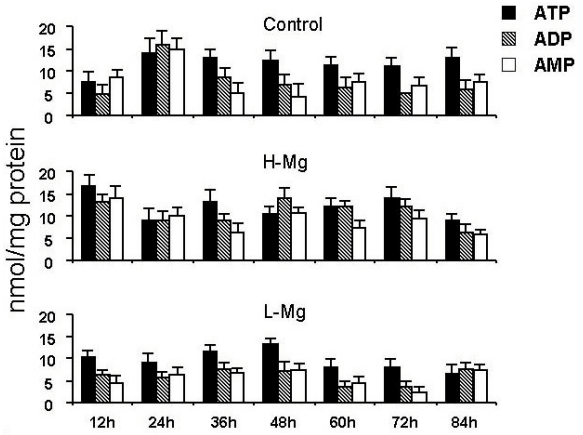


**Figure 3.** Cell adenine nucleotides contents of HC-11 cells during growth in control, high and low magnesium media. At the indicated time intervals adenine nucleotides were assayed in acidic extracts by reverse phase HPLC. Values are means +/- SEM of 3 separate experiments. Differences between adenine nucleotide levels in the three cell types were not statistically significant.

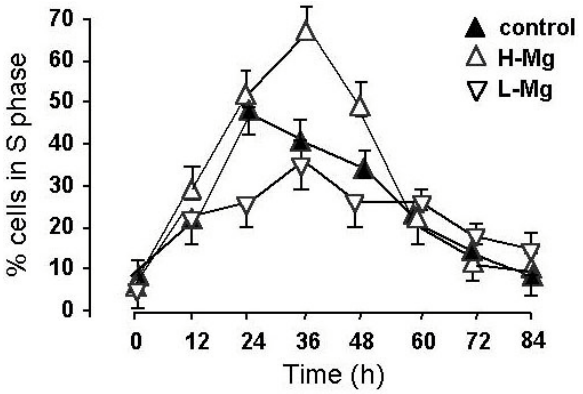


**Figure 4.** Percentage of cells in S phase of the cell cycle of HC-11 cells during growth in control, high and low magnesium media. At the indicated time intervals cell cycle distribution was analysed by cytofluorimetry. The percentage of cells in S phase at time 0 were considered that of 84h cultures. Data are means +/- SEM of 3 separate experiments.

We have previously shown that total Mg content decreased during the differentiation of HL-60 cells and that such decrease, involving primarily mitochondrial and cytoplasmic pools, was due to a parallel decrease of adenine nucleotides (24). We therefore characterized whether the increase of Mg content which occurred in proliferating cells was due to a parallel increase of adenine nucleotides. To this aim we measured ATP, ADP and AMP content during the growth of control and H-Mg or L-Mg cells. To avoid depletion of substrates for energy metabolism the culture medium was renewed every 24h rather than every 48h; control experiments showed that such changes did not affect the growth rates previously shown in Figure 1. Data obtained from 12 to 84h showed that there were no dramatic modifications of the cellular levels of ATP and ADP or AMP, nor did the levels of the nucleotides follow a bell-shaped pattern which could explain changes of Mg content occurring at the same time points (Figure 4).

The increase of cell total Mg observed during proliferation correlates with the exponential phase of growth; therefore, we thought it was of interest to measure cell cycle distribution in our three cell cultures and to compared it with corresponding changes in intra cellular Mg content. Figure 4 shows that percentage of cells in S phase increased at 12h in all cell populations, peaking at 24h in control cells (about 40% cells in S phase), and at 36h in H-Mg (about 60% cells in S phase) or L-Mg cells (about 30% cells in S phase). The percentage of cells in S phase then decreased at 72h in all cell types. From a quantitative view point it was therefore evident that control and H-Mg cells exhibited a more pronounced proliferation pattern compared to L-Mg cells, similar to what observed when measuring cell number in Figure 1. Moreover, the percentage of cells in the S phase reproduced concomitant changes in intra cellular Mg content, with both parameters increasing and decreasing sharply in control and H-Mg cells while exhibiting more prolonged increases in L-Mg cells. These data further confirm an essential role of Mg in cell proliferation and suggest a specific role for Mg in the S phase of the cell cycle.

The S phase is characterized by DNA duplication, accompanied by increased protein synthesis; therefore, we measured total nucleic acids content in control and H-Mg or L-Mg cell at various times of growth in culture. Results showed a bell-shaped pattern according to which nucleic acids increased at 12h, peaked at 36h and then returned to baseline values. At 36h nucleic acids increased 63% in control cells (from 5.5 to 9.0 micro g/$10^6$ cells), 100% in H-Mg cells (from 7.0 to 14 micro g/$10^6$ cells), 29% in L-Mg cells (from 4.5 to 5.8 micro g/$10^6$) (Figure 5). The time courses and net levels of nucleic acid closely reproduced the changes in intra cellular Mg occurring under comparable experimental conditions, thereby providing an additional evidence that Mg was involved in proliferation-linked events. Comparisons between control and H-Mg or L-Mg cells therefore show that total intra cellular Mg increases during proliferation, regardless of extra cellular Mg availability, and that such increase coincides with the S phase and the possible involvement of Mg in DNA duplication and transcription. While extending similar previous reports (9, 27) these findings offer a more comprehensive picture of the relationships between intra cellular Mg content and biochemical or kinetic events in cells that proliferate under conditions of physiologic or abnormal Mg availability.

### 4.2. Magnesium and membrane potential and pH

Data shown in the preceding sections showed that cell proliferation was accompanied by Mg accumulation in both control and H-Mg or L-Mg cells; moreover, a correlation existed between proliferation and Mg accumulation, both processes following a pattern of magnitude that was control cells equal to H-Mg cells, more than L-Mg cells. We attempted to characterize the mechanisms underlying proliferation-dependent Mg accumulation in the

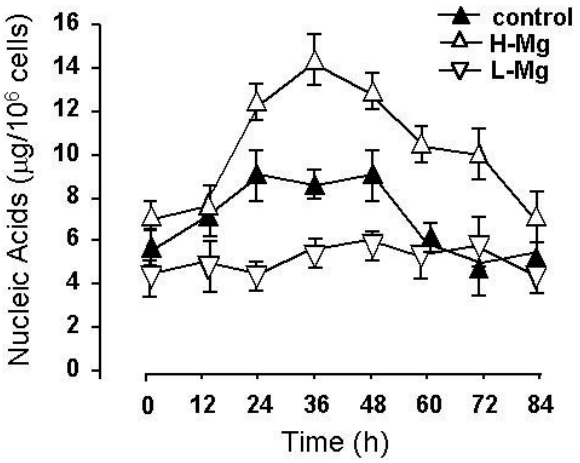


**Figure 5.** Total nucleic acid contents of HC-11 cells during growth in control, high and low magnesium media. Nucleic acids were assayed in chloroform/methanol extracts at each time points. Data are means +/- SEM of 3 separate experiments.

three cell lines. In addressing this issue we considered that membrane potential and pH are important determinants of cell proliferation and Mg movements. Changes in membrane potential and pH have been described as very early events in the induction of mitosis (28, 29). On the one hand, hyper polarization will favour Mg influx from the extra cellular medium by increasing intra cellular levels of negatively charged species like $Cl^-$; on the other hand, depolarization is expected to decrease intra cellular Mg by diminishing its influx and/or by increasing its efflux. Finally, acidic pH will oppose to Mg influx whereas basic pH will facilitate it. We therefore characterized whether membrane potential and pH changed during proliferation of HC-11 cells and how these changes influenced Mg content of control and H-Mg or L-Mg cells. Figure 6A shows cytofluorimetric analyses of membrane potential and pH in the three cell types at 24 h culture, a time when they all were in an exponential phase of growth. Results show that, in comparison to control cells, H-Mg cells were characterized by membrane depolarisation. Such depolarisation may have the role of counteracting chemical gradient due to high extra cellular Mg, thereby preventing Mg from accumulating in excess of metabolic and proliferation requirements. Accordingly, H-Mg cells exhibited the same Mg accumulation and proliferation rate of control cells that were grown in ~50-fold lower extra cellular Mg. Different results were obtained with L-Mg cells, characterized by membrane hyper polarization compared to control cells. Such hyper polarization may have the role of facilitating Mg influx, thus explaining the moderate but significant accumulation of Mg and increase of proliferation which characterized these cells, in face of their very low availability in extra cellular Mg. Similar measurements of intra cellular pH showed that, in comparison to control cells, H-Mg cells were characterized by a remarkable shift toward acidic pH (Figure 6B). This may serve an additional mechanism to avoid excess Mg entry during proliferation of these cells. Again, different results were obtained with L-Mg cells, which exhibited a trend toward an alkaline pH. Although less evident than the concomitant increase of membrane potential, such alkalinization may be of some importance in facilitating Mg entry, and hence proliferation, in these cells.

## 5. CONCLUSIONS

We have shown that cell proliferation is accompanied by important changes of total intra cellular Mg, regardless of how much extra cellular Mg is available. Control and H-Mg cells show a time-dependent increase of total Mg which is evident also when changes in cell volume are taken into account. This is less evident in the case of L-Mg cells, due to a combination of factors like the very low availability of extra cellular Mg, the slow proliferation rate, and the rather modest (although significant) increase of total Mg/mg protein. Importantly, the increase of cell Mg cannot be attributed to any consistent increase of adenine nucleotides, which in fact remained stable during cell proliferation nor were appreciably influenced by extra cellular Mg. Our results clearly show that total cell Mg increases during the S phase of the cell cycle, concomitant with DNA duplication and transcription. Finally, we have shown that membrane potential or pH might modulate intra cellular Mg content; in particular, membrane potential favors Mg influx and retention in the slow proliferating L-Mg cells, whereas acidic pH might serve to preventing excess Mg influx in the high-proliferating H-Mg cells. These data offer novel information on ionic events associated with cell proliferation in the presence of both normal and very high or very low extra cellular Mg, and may be relevant to diseased states possibly linked to Mg changes in biologic fluids. Our results also prompt further studies to elucidate the precise role of free *vs* bound Mg in modulating proliferation events characterized by changes of total intra cellular Mg like those described in our present study.

## 6. ACKNOWLEDGMENTS

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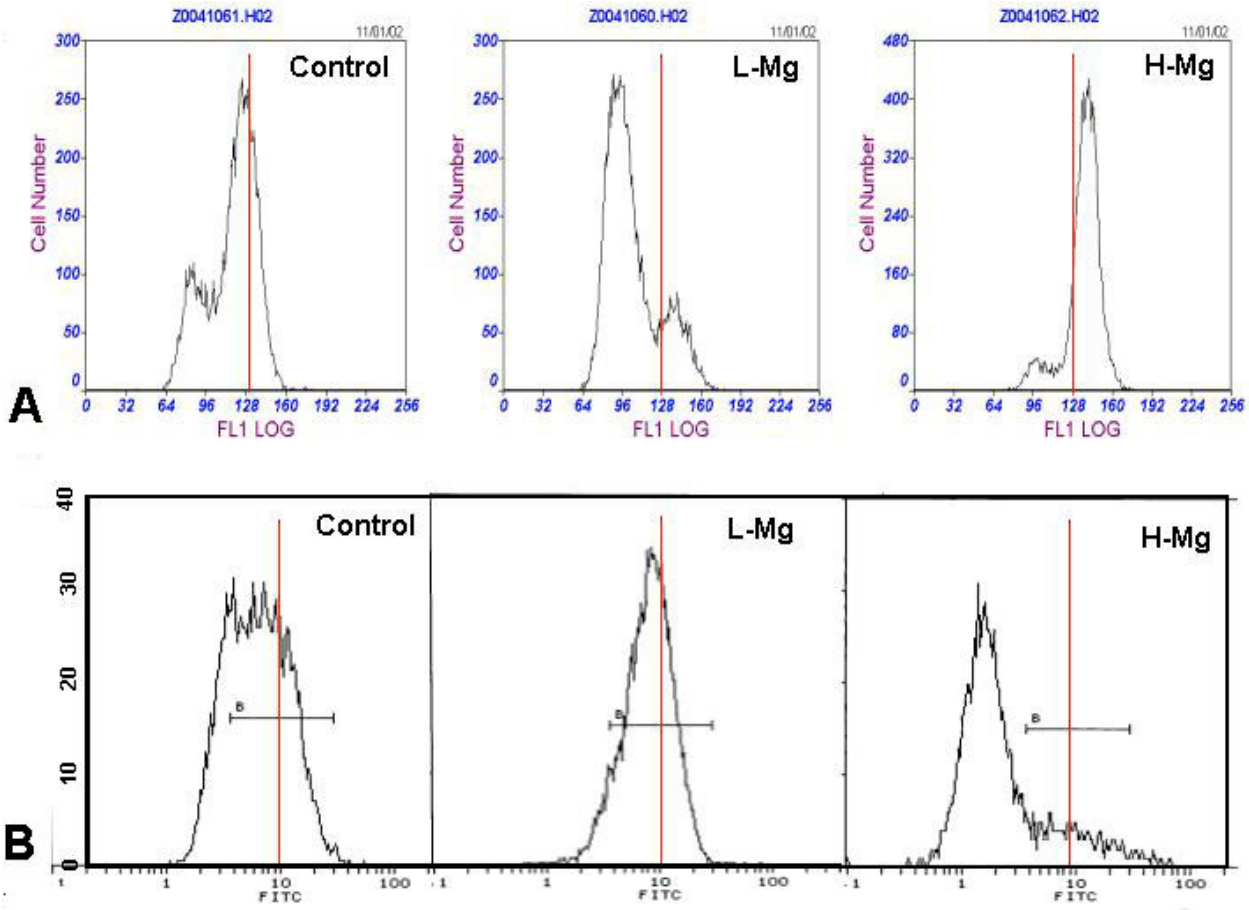


**Figure 6.** Flow cytofluorimetric measurements of Membrane potential and intra cellular pH of HC-11 cells in control, high and low magnesium media. Membrane potential was assayed on DiSBA-$C_4$-(3)–loaded cells at 24h of culture, that is during the exponential phase of cell growth (panel A). Diagrams show the distribution of cells according to fluorescence. Depolarisation induces an increased fluorescence and corresponds to a shift to the right; hyper-polarisation corresponds to decreased fluorescence and shift to the left. pH was measured on BCECF–loaded cells at the same time points (panel B). Increase in fluorescence, (shift to the right), corresponds to more alkaline pH; decrease in fluorescence (shift to the left), indicates more acidic pH. Data from typical experiments repeated two times with similar results.

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[1] **Footnote:** Magnesium is indicated by its chemical symbol (Mg) when reference is made to total elemental content, without specifying whether Mg is free, bound or uncharged. $Mg^{2+}$ refers only to the element in the free form in solution and $[Mg]_i$ or $[Mg]_o$ refers to intra cellular or extra cellular ionised Mg concentration, respectively.

**Key Words**: Cell cycle, Adenine nucleotides, Nucleic acids, Membrane potential, pH, Atomic absorption spectrometry

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