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# EGF stimulates $Mg^{2+}$ influx in mammary epithelial cells

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ABSTRACT

Magnesium is well established as a fundamental factor that regulates cell proliferation. However, the molecular mechanisms linking mitogenic signals, extracellular magnesium availability and intracellular effectors are still largely unknown. In the present study we sought to determine whether EGF regulates magnesium homeostasis in normal HC11 mammary epithelial cells. To this end, we measured $Mg^{2+}$ and $Ca^{2+}$ fluxes by confocal imaging in live cells loaded with specific fluorescent ion indicators (Mag-Fluo-4 and Fluo-4, respectively).

EGF stimulation induces a rapid and sustained increase in intracellular $Mg^{2+}$, concomitantly with a rise in intracellular calcium. The increase in intracellular $Mg^{2+}$ derives from an influx from the extracellular compartment, and does not depend on $Ca^{2+}$. On the contrary, the increase in intracellular $Ca^{2+}$ derives from intracellular stores, and is impaired in the absence of extracellular magnesium. Inhibition of the EGF receptor tyrosine kinase by Tyrphostin AG1478 markedly inhibits EGF-induced $Mg^{2+}$ and $Ca^{2+}$ signals.

These findings demonstrate that not only does $Mg^{2+}$ influx represent an important step in the physiological response of epithelial cells to EGF, but unexpectedly the EGF-induced $Mg^{2+}$ influx is essential for the $Ca^{2+}$ signal to occur.



## 1. Introduction

Intracellular magnesium is well recognized as a key factor for the wide variety of reactions involved in the response to mitogenic stimuli [1]. The correspondence between cell magnesium content, the rate of protein synthesis, the later onset of DNA synthesis and the consequent proliferation rate is well established, as is the $G_0/G_1$ growth arrest induced by low magnesium availability [2]. At present, however, very little is known about the molecular mechanisms coupling growth factor receptor activation and extracellular magnesium availability to intracellular effectors and downstream events.

Intracellular magnesium content, in the free ($Mg^{2+}$) or bound (Mg) form, is tightly regulated in the face of varying extracellular availability. Indeed, during the exponential phase of cell growth, total intracellular magnesium rises regardless of extracellular magnesium concentration [3]. This strongly suggests that there must be active mechanisms to recruit magnesium, and these are likely to be triggered by mitogenic signals. Early findings suggested that a mobilization of $Mg^{2+}$ is a shared response to different mitogenic stimuli in a variety of cell types [4–8], but whether $Mg^{2+}$ could act as a second messenger in intracellular signaling has long remained controversial. In contrast to what occurs for $Ca^{2+}$, detection of clear-cut $Mg^{2+}$ fluxes is particularly challenging. In fact, $Mg^{2+}$ fluctuations are modest in comparison to its high and well-buffered intracellular physiologic concentration (in the sub-millimolar range). The increase in total intracellular magnesium observed in growing cells or tissues mainly reflects an increase in the bound pool, rather than in free $Mg^{2+}$. Modifications in the bound pool are coupled to cell cycle-associated metabolic transitions such as increase in ATP, protein and DNA syntheses [9].

Recently, the involvement of $Mg^{2+}$ in T-cell receptor (TCR) signaling was proven [10]. It was shown that $Mg^{2+}$ influx has a crucial role in transducing the signal that leads from TCR stimulation to cell activation. Significantly, the same study also pointed out that magnesium acts synergistically with calcium to promote cell activation, which is in marked contrast with the classical view that the two ions behave as antagonists.

*Abbreviations:* BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; MagT1, magnesium transporter 1; NMDG, N-methyl-D-glucamine; PLC, phospholipase C; SLC, solute carrier; TCR, T-cell receptor; TRPM, transient receptor potential melastatin type.

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In the present study we sought to ascertain the role of magnesium in the response of epithelial cells to EGF stimulation. We used an in vitro cellular model that has been extensively characterized in our laboratory. We already described HC11 mammary epithelial cells in terms of proliferative behavior, magnesium content and magnesium channel expression in conditions of different magnesium availability [3,11–13]. HC11 cells express the magnesium channels TRPM6, TRPM7 and MagT1, which are responsible for $Mg^{2+}$ influx from the extracellular compartment, and the $Na^+$/$Mg^{2+}$ exchanger, most likely SLC41A1, which mediates $Mg^{2+}$ efflux from the cell [13]. HC11 cells are spontaneously immortalized mammary epithelial cells that are responsive to EGF [14]. Therefore, we measured ion fluxes induced in HC11 cells by acute EGF treatment; in particular, we focused on the relationship between calcium and magnesium fluxes, and their mutual dependence.

## 2. Materials and methods

### 2.1. Cell culture

Reagents for cell culture were from Euroclone (Pero, Milan, Italy). Recombinant EGF was purchased from PeproTech (London, UK). The HC11 cell line was clonally derived from a spontaneously immortalized mammary epithelial cell culture originally established from a midterm pregnant BALB/c mouse [14]. HC11 cells were cultured in MEM medium with Earle's salts, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 10 ml/l penicillin/streptomycin solution at 37 °C and 5% $CO_2$. Before EGF stimulation, cells were starved in FBS-free medium for 24 h.

### 2.2. Live confocal imaging

Fluo-4-AM, Mag-Fluo-4-AM, BAPTA-AM and Pluronic F-127 were purchased from Invitrogen (Monza, Italy). Cells were plated on 35-mm microscopy dishes (μ-dish, Ibidi, Martinsried, Germany) and incubated in complete medium for 24 h before starvation. Following a 24-h starvation in FBS-free medium, subconfluent cultures were analyzed by live confocal imaging after loading with Fluo-4-AM or Mag-Fluo-4-AM for $Ca^{2+}$ or $Mg^{2+}$ measurements, respectively. In selected experiments, cells were pretreated with 2 μM Tyrphostin AG1478 (Sigma–Aldrich, Milan, Italy) for 90 min. Cell loading was performed as previously described [13]. Where indicated, BAPTA-AM (5 μM) was added to the loading buffer. After loading and immediately before imaging, cells were transferred to a $Na^+$, $Ca^{2+}$ and $Mg^{2+}$-free buffer (NMDG buffer), prepared by equimolar substitution of $Na^+$ with N-methyl-D-glucamine and the remaining components as for the loading buffer.

Cytosolic fluorescence signals were recorded for 5 min at a confocal laser scanning system (TCS-SP2, Leica Microsystems, Wetzlar, Germany). The baseline was monitored for 30 s, then $MgSO_4$ was added dropwise in order to achieve the desired final concentration (from 0 to 40 mM, depending on the experiment). Where indicated, EGF (10 ng/ml) was added together with $MgSO_4$. Changes in intracellular $Ca^{2+}$ or $Mg^{2+}$ levels at the single cell level were estimated by the mean fluorescent increment $\Delta F/F$ [15]. Image analysis was performed by Leica Confocal Software and 10 representative cells were examined in each microscopic field. Experiments were repeated independently three times with similar results.

### 2.3. Statistical analysis

Data are expressed as mean ± SE. Significance was determined by unpaired *t* test; alternatively, one-way analysis of variance (ANOVA) was performed to assess differences between multiple sets of data. When significant values were found ($P < 0.05$), post hoc significance of means was calculated by the Tukey's multiple comparison test. All statistical calculations were performed by Prism 4 software (GraphPad Software Inc., San Diego, CA).

## 3. Results

It is recognized that detection of basal $Mg^{2+}$ fluxes in several cell types can be accomplished only by increasing the otherwise modest plasma membrane Nernst potential (resulting from ≈1.0 mM $[Mg^{2+}]_{out}$ *vs.* ≈0.5 mM $[Mg^{2+}]_{in}$), and inhibiting the $Na^+$-dependent $Mg^{2+}$ efflux, which counteracts any rise in cytosolic $Mg^{2+}$ [15]. Therefore HC11 cells were incubated in a $Na^+$-free buffer (NMDG buffer, see Section 2.2) and extracellular magnesium concentration was increased above the physiological concentration.

First of all, we characterized the basal magnesium influx capacity of HC11 cells. As shown in Fig. 1, $Mg^{2+}$ influx was barely detectable upon addition of a physiological $Mg^{2+}$ concentration (1 mM), but the influx rate could be increased by extracellular $MgSO_4$ addition up to 40 mM. As a consequence, the intracellular $Mg^{2+}$ concentration displayed a sustained increase reaching a plateau within 5 min.

Next, we investigated whether EGF affected the basal $Mg^{2+}$ influx capacity of HC11 cells (Fig. 2). When EGF (10 ng/ml) was added together with $MgSO_4$, $Mg^{2+}$ influx was potentiated at all tested concentrations: EGF increased both the initial influx rate (Fig. 2A) and the total fluorescence increment within 5 min from stimulation (Fig. 2B). In the following experiments, a 20 mM concentration was chosen as the best experimental condition to detect a significant $Mg^{2+}$ influx without kinetics saturation. EGF stimulation in the absence of extracellular magnesium did not induce any appreciable change in the intracellular fluorescence (Fig. 2C), which implies that the increase in intracellular $Mg^{2+}$ derives from influx from the extracellular compartment, rather than from an intracellular release. On the contrary, absence of extracellular $Ca^{2+}$ did not affect the EGF-induced $Mg^{2+}$-influx, as all experiments were conducted in a $Ca^{2+}$-free buffer (see Section 2.2). Likewise, chelation of intracellular $Ca^{2+}$ by BAPTA did not significantly change the observed EGF-induced increase in intracellular $Mg^{2+}$ (Fig. 2C).

EGF is known to induce an increase in the intracellular $Ca^{2+}$ concentration. Indeed, Fig. 3 shows that EGF stimulation of HC11 cells did provoke a rapid increase in intracellular $Ca^{2+}$ concentration, which returned to basal levels within minutes. Such increase was observed in a $Ca^{2+}$-free buffer (see Section 2.2), but was completely abolished in the presence of the intracellular $Ca^{2+}$ chelator BAPTA, which proves that $Ca^{2+}$ is released from intracellular stores. Importantly, removing $Mg^{2+}$ from the extracellular medium eliminated the EGF-induced $Ca^{2+}$ signal.

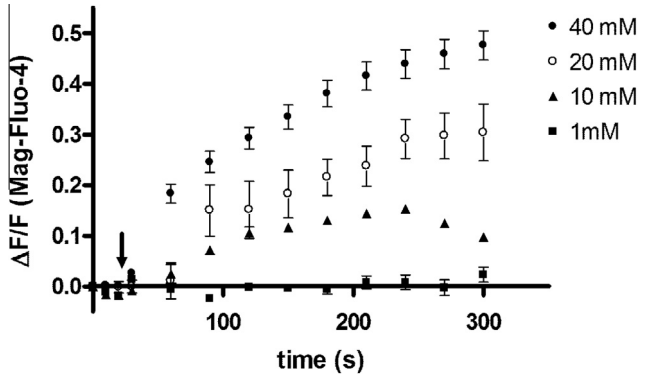


**Fig. 1.** Basal $Mg^{2+}$ influx capacity of HC11 cells. Mag–Fluo-4-loaded cells were incubated in NMDG buffer (see Section 2.2). At $t = 30$ s (arrow) the indicated $Mg^{2+}$ concentration was added to the buffer. The fluorescence signal was collected by confocal live imaging and single-cell fluorescence was evaluated by image analysis. The time course of mean fluorescence increment ($\Delta F/F$) of 10 cells ± SE from a representative experiment is reported ($n = 3$).

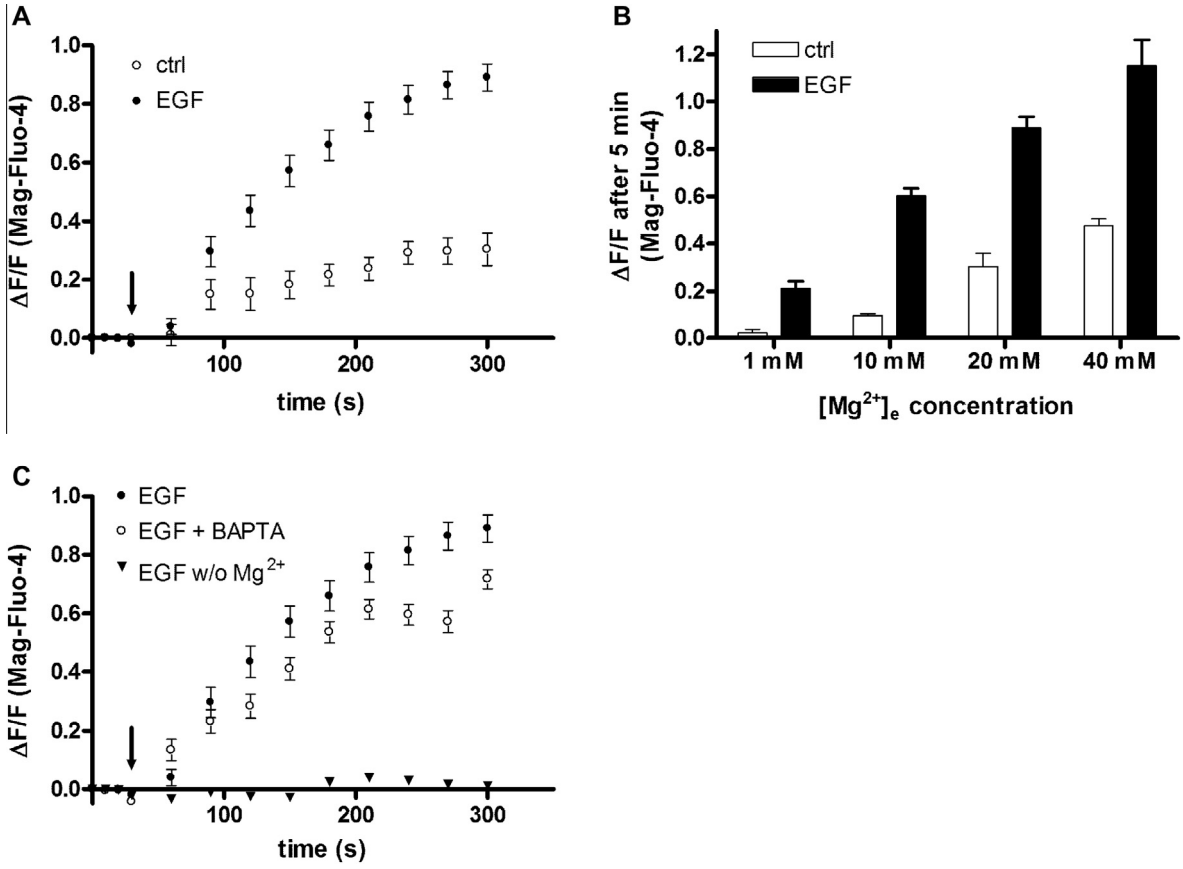


**Fig. 2.** Stimulation of $Mg^{2+}$ influx induced by EGF in HC11 cells. Mag–Fluo-4-loaded cells were incubated in NMDG buffer (see Section 2.2). The fluorescence signal was collected by confocal live imaging and single-cell fluorescence was evaluated by image analysis. The mean fluorescence increment ($\Delta F/F$) of 10 cells ± SE from a representative experiment is reported ($n = 3$). (A) Time course of the mean fluorescence increment ($\Delta F/F$) upon addition (arrow) of 20 mM $MgSO_4$ in the presence (solid circles) or absence (open circles) of EGF (10 ng/ml). (B) Mean fluorescence increment as measured 5 min from addition of the indicated $Mg^{2+}$ concentration with (black) or without (white) EGF. (C) Time course of the mean fluorescence increment ($\Delta F/F$) upon addition (arrow) of EGF (10 ng/ml) and 20 mM $MgSO_4$ in the absence (solid circles) or presence of the $Ca^{2+}$ chelator BAPTA (open circles), or of EGF alone in the absence of external $Mg^{2+}$ (inverted triangles).

Altogether, these results prove that EGF stimulation causes an increase in the intracellular $Mg^{2+}$ concentration by potentiating $Mg^{2+}$ influx from the extracellular compartment. Crucially, the EGF-induced $Mg^{2+}$ influx does not depend on the concomitantly induced $Ca^{2+}$ signal, as it is measurable both in the absence of extracellular and intracellular $Ca^{2+}$. On the other hand, the EGF-induced $Ca^{2+}$ signal appears to be strictly dependent on the presence of extracellular $Mg^{2+}$.

The EGF signal transduction pathway involves EGF binding to its receptor (EGFR), which stimulates the intrinsic tyrosine kinase activity of the EGFR and hence initiates the signaling cascade. Pretreatment of HC11 cells with Tyrphostin AG1478, a specific inhibitor of the EGFR tyrosine kinase, abrogated the $Mg^{2+}$ influx and significantly reduced the $Ca^{2+}$ signal induced by EGF stimulation (Fig. 4A and B, respectively). In conclusion, changes of both ion concentrations are downstream events that follow EGFR activation.

## 4. Discussion

The EGF signal transduction pathway is an established paradigm for growth-factor induced cell proliferation wherein $Ca^{2+}$ plays a primary role as a second messenger. The results we here present add unexpected twists to an old story. To our knowledge, we are the first to show that in mammary epithelial cells, together with the well-known release of $Ca^{2+}$ from intracellular stores, EGF also stimulates $Mg^{2+}$ influx from the extracellular milieu (Fig. 1). Such influx is proportional to the extracellular $Mg^{2+}$ concentration (Fig. 2A and B) and is independent both from extracellular and intracellular $Ca^{2+}$ (Fig. 2C).

These findings perfectly fit into the long thread of evidence pointing to magnesium as a crucial regulating factor of cell growth and proliferation, and provide new tiles for the identification of the long-sought molecular link between mitogenic factors and magnesium availability (outside), and second messengers and effectors (inside). The difference in the observed kinetics of EGF-induced $Mg^{2+}$ influx as opposed to the concomitant change in the intracellular $Ca^{2+}$ concentration might seem to corroborate the classical view that $Mg^{2+}$ would act as a chronic modulator of cell function in contrast to the acute regulation elicited by second messengers such as $Ca^{2+}$ [16]. Nonetheless, we present unforeseen evidence that magnesium is indispensable for modulating the $Ca^{2+}$ signal induced by EGF stimulation, as such signal is abolished in the absence of extracellular magnesium (Fig. 3). These results imply that $Mg^{2+}$ influx is situated upstream the release of $Ca^{2+}$ in the EGF signal transduction pathway, and thus magnesium might be truly considered a second messenger of receptor-mediated signals.

A similar scenario was described in T lymphocytes, where a signaling role for $Mg^{2+}$ was demonstrated in T-cell activation following TCR engagement [10]. It was proven that TCR stimulation produced a $Mg^{2+}$ influx necessary for downstream PLCγ1 activation leading to $Ca^{2+}$ release; interestingly, $Ca^{2+}$ release following PLCβ activation in other cell types did not seem to require magnesium. The EGF signal transduction pathway in epithelial cells similarly requires PLCγ1 activation to trigger $Ca^{2+}$ release [17], and our results show that the presence of magnesium is essential for the EGF-induced $Ca^{2+}$ signal, which suggests the involvement of the same molecular players. At present we can only speculate as to

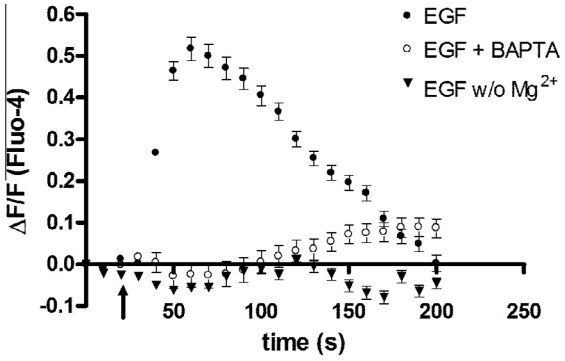


**Fig. 3.** $Ca^{2+}$ fluxes induced by EGF in HC11 cells. Fluo-4-loaded cells were incubated in NMDG buffer (see Section 2.2). The fluorescence signal was collected by confocal live imaging and single-cell fluorescence was evaluated by image analysis. Time course of the mean fluorescence increment ($\Delta F/F$) of 10 cells ± SE from a representative experiment is reported ($n = 3$). At $t = 30$ s (arrow), EGF (10 ng/ml) was added in the presence (20 mM, solid circles) or absence (inverted triangles) of external $Mg^{2+}$. The effect of the $Ca^{2+}$ chelator BAPTA was assessed in the presence of 20 mM $MgSO_4$ (open circles).

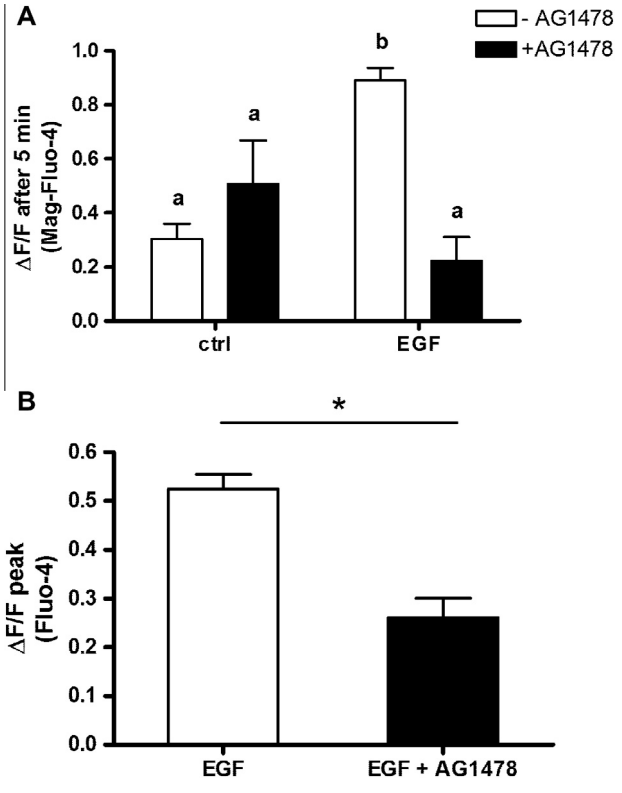


**Fig. 4.** Effect of EGFR inhibition on EGF-induced ion fluxes. Mag–Fluo-4 or Fluo-4-loaded cells were incubated in NMDG buffer for $Mg^{2+}$ (A) or $Ca^{2+}$ (B) measurements, respectively (see Section 2.2). The fluorescence signal was collected by confocal live imaging and single-cell fluorescence was evaluated by image analysis. Maximum fluorescence increment upon EGF treatment in the presence of 20 mM $MgSO_4$ with (black) or without (white) Tyrphostin AG1478 pretreatment (2 μM, 90 min) is reported. Data sharing the same letter are not statistically different as assessed by Tukey's multiple comparison test. $^*P < 0.001$ (Student's $t$ test).

which of the magnesium channels expressed in HC11 cells [13] takes part in the described process. Available information does not rule out a putative role for any of them. MagT1 has been involved in TCR signaling *via* PLCγ1 [10]; TRPM6 expression and activity are upregulated by EGF in renal epithelial cells [18]; finally TRPM7, which is thought to function as a heterotetramer formed by TRPM6 and TRPM7 subunits [19], has been most convincingly associated to cell proliferation in a wide variety of cell types [20]. Selective knockdown by gene silencing is ongoing to ascertain the molecular identity of the channel(s) that mediate the EGF-induced $Mg^{2+}$ influx.

In conclusion, in this paper we have provided evidence that $Mg^{2+}$ influx might represent an important step in the physiological response of epithelial cells to EGF, and likely to the resulting induction of proliferation. The magnesium channel that mediates this influx as well as the intracellular effectors of the triggered signal transduction pathway remain to be elucidated. Identification of the molecular players coupling EGFR tyrosine kinase activation to $Mg^{2+}$ influx, and understanding how the latter relates to $Ca^{2+}$ signal, may ultimately lead to validation of novel therapeutic targets for diseases characterized by altered cellular growth, *e.g.* cancer.

## Conflict of interest statement

The authors declare no conflict of interest.

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