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Biphasic Effect of Extra-reticular Mg^{2+} on Hepatic G6P Transport and Hydrolysis

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Abstract

Magnesium ions (Mg^{2+}) play a key role in regulating hepatic cellular functions and enzymatic activities. In the present study, we report a concentration-dependent effect of cytosolic Mg^{2+} on G6P1 and pyrophosphate (PPi) transport and hydrolysis in digitonin-permeabilized rat hepatocytes. The stimulatory effect of Mg^{2+} on G6P is specific but biphasic, with a maximal effect at a concentration of 0.25mM, whereas the effect on PPi increases in a dose-dependent manner. Both effects can be abolished by addition of EDTA to the system. Addition of taurocholate, histone-2A, alamethicin or A23187 to the incubation system results in a marked decrease in the Mg^{2+} concentration present within the endoplasmic reticulum lumen. Under these conditions, the stimulatory effect of extra-reticular Mg^{2+} on G6P transport and hydrolysis is abolished. Taken together, these data suggest that cytosolic Mg^{2+} stimulates G6P transport by acting at the level of the substrate binding site of the G6Pase enzymatic complex or the surrounding phospholipid environment. The effect, which is lost when G6P has readily access to the ER lumen, requires physiological endoplasmic reticulum Mg^{2+} content.

Keywords

Mg^{2+}; G6P; G6Pase; pyrophosphate; endoplasmic reticulum; hepatocyte

INTRODUCTION

Magnesium (Mg^{2+}) is the second most abundant cation after potassium [1]. In the majority of mammalian cells, total Mg^{2+} concentration ranges between 16 to 18mM within nucleus, mitochondria and endo-sarco-plasmic reticulum [1]. In the cytoplasm, Mg^{2+} is mostly in a form of a complex with ATP or other phosphonucleotides (about 4–5mM, [**2]**), so that 0.5–0.7mM is actually in a free form ($[Mg^{2+}]_{i,}$ [1,3]). As our understanding of cellular Mg2+ homeostasis has improved over the years, the relevance of Mg^{2+} in regulating cell cycle, channel function and various cellular enzymes has also increased [4]. One of the last counts indicates more than 300 enzymes, channels, and kinases as being regulated directly or indirectly by Mg^{2+} [4]. The list includes enzymes regulating glycolysis and glucose homeostasis [4], as well as regulatory enzymes such as adenylyl cyclase [4]. The existence of a 'link' between glucose utilization and Mg^{2+} homeostasis is supported by the observation that liver cells mobilize Mg^{2+} in addition to glucose in response to the exogenous administration [5] or the endogenous release [6] of glucagon or catecholamine. Technical limitations, however, have

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hampered our ability to properly relate changes in total or free cellular Mg^{2+} content with variations in Mg^{2+} content within specific cellular compartments, *de facto* preventing us from establishing cause-effect correlation between hormone-mediated Mg^{2+} fluxes at the cellular level with changes in Mg^{2+} concentration and activity of specific enzymes within cellular compartments.

Glucose-6-phosphatase (G6Pase, EC 3.1.3.9) is an enzymatic complex operating within the endoplasmic reticulum (ER) of liver [7], kidney [8] and pancreatic beta-cells [9]. Because it catalyzes the hydrolysis of glucose 6-phosphate (G6P) to glucose and phosphate (Pi), this enzyme has a key role in regulating glycolysis and glucose homeostasis [10]. Structurally, G6Pase presents nine transmembrane helices embedded in the ER membrane [11,12] with the catalytic site of the hydrolase being located within the ER lumen [13]. Thus, to be hydrolyzed the substrate G6P must enter the ER via a specific transport mechanism [11]. Despite extensive studies, the structure and operation of this enzymatic complex are still debated. Two models have been proposed. In the *substrate-transport model*, G6P enters the ER lumen via a specific transporter (T1) distinct from the hydrolase. In this model, T1 represents the rate-limiting factor for the G6Pase system [14]. The *conformational flexibility substrate-transport model*, instead, proposes that in addition to hydrolytic activity the G6Pase enzyme would possess a hydrophilic region at its cytoplasmic side responsible for substrate binding. Binding of the substrate to this region would result in a conformation change and delivery of the substrate to the intra-luminal catalytic site. According to this model, the substrate binding site and the hydrolytic site of G6Pase are two parts of the same protein, and the enzyme is not specific for a particular substrate [13]. In both models, the bio-products of G6P hydrolysis (i.e. glucose and Pi) are released into the cytoplasm via two additional, specific transport mechanisms [10,13–15].

Pyrophosphate (PPi) can also be hydrolyzed by the G6Pase system [13]. However, the transport mechanism delivering PPi to the ER lumen is reputed to be distinct from that involved in G6P transport [13].

In the present study, collagenase-dispersed hepatocytes permeabilized by digitonin were used to test the hypothesis that changes in cytoplasmic or endo-luminal Mg^{2+} concentration could modulate G6P hydrolysis. The reported results suggest that changes in cytoplasmic Mg^{2+} level can affect the rate of G6P hydrolysis by acting on the G6P transport mechanism. The physio-pathological relevance of these results is discussed.

MATERIALS AND METHODS

Materials

All chemicals were of the purest analytical grade (Sigma, St. Louis, MO). Di-sodium salt hydrate G6P and M6P were used in the study. Collagenase (CLS-2) was from Worthington Biochemical Corporation (Lakewood, NJ).

Hepatocytes Isolation

Hepatocytes were isolated as previously described [6]. Fed male Sprague–Dawley rats (230–250 g body weight) were anesthetized by i.p. injection of saturated sodium pentobarbital solution (65mg/ml). The portal vein was cannulated, and hepatocytes were isolated by collagenase digestion. Following isolation, hepatocytes were washed twice in a medium containing 120mM NaCl, 3mM KCl, 1.2mM KH_2PO_4, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 10mM HEPES, 10mM glucose, 12mM $NaHCO_3$ (pH 7.35) equilibrated with O2: CO2 (95:5 v/v), and resuspended therein, at a final volume of 30 ml. After passage through a Percoll gradient [6] to maximize cell viability, the hepatocytes were resuspended in the medium described above,

at a final concentration of 1×10^5 cells/ml. Trypan blue exclusion test indicated a viability of 95±3% (n=10), which did not change significantly over 2 hours (94±4%, n= 10).

Hepatocyte Permeabilization

Five ml aliquots of hepatocyte suspension were withdrawn and gently sedimented at 600 g × 1 min. The cell pellet was then resuspended in a *cytosol-like* medium containing 20mM NaCl, 100mM KCl, 10mM MOPS (pH 7.2) plus 50μg/ml digitonin [16]. Cell permeabilization, assessed by trypan blue exclusion test, was attained within 5 min at room temperature (96±2% trypan blue positive, n=10). Digitonin-permeabilized cells were sedimented at 1,200 g × 1 min and washed twice in the cytosol-like medium indicated above to remove cytoplasmic components. The final pellet was resuspended in the same medium at the final concentration of 1×10^5 cells/ml, and used immediately.

In a separate set of experiments, digitonin-permeabilized hepatocytes were incubated for 15 min at room temperature in the presence of 1mM (final concentration) taurocholate to attain endoplasmic reticulum (ER) permeabilization [17]. ER-permeabilized hepatocytes were then washed twice and resuspended in a similar volume of cytosol-like medium as indicated previously, and used immediately.

G6Pase Activity

Digitonin-permeabilized or digitonin-taurocholate-permeabilized hepatocytes were incubated at the final concentration of 200–250μg/ml in the cytosol-like medium described above in the presence of varying concentrations of Mg^{2+} (as $MgCl_2$), at 37°C. Hydrolytic activity was initiated by addition of varying concentrations of G6P, PPi or M6P, and carried out for at least 45 min. All the substrates were dissolved in the cytosol-like buffer devoid of magnesium. Starting from time = 0 min, 1 ml aliquots of the incubation mixture were removed at 15 min intervals, and the hydrolysis activity stopped by rapid addition of TCA (5% final concentration). The acid mixture was then sedimented at 1,500 rpm for 10 min in a refrigerated Beckman B6J centrifuge. The supernatant was removed and the amount of Pi present was measured spectrophotometrically according to the Fiske and SubbaRow assay [18] at 700 nm in an Agilent 8453 spectrophotometer equipped with a computerized data acquisition program.

In selected experiments, histone 2A (250μg/ml or isodose with the amount of protein present in the system, [19]) or alamethicin (3μg/ml, [19]) were added directly to the incubation mixture containing digitonin-permeabilized hepatocytes to enhance ER membrane permeability to G6P [9,19]. In a separate set of experiments, the ionophore A23187 was added at the final concentration of 2μg/ml to mobilize Mg^{2+} from ER [20] without altering ER membrane permeability to G6P.

For the experiments involving EDTA to chelate extra-reticular added Mg^{2+} to 0mM, a computational program [21] was used to determine the correct amount of chelating agent to add to the incubation system.

G6P Transport Assay

Aliquots of digitonin-permeabilized cells were incubated in the presence of varying Mg^{2+} concentrations at the final concentration of 200–250μg protein/ml. After 2 min equilibration, 5mM G6P labeled with 1μCi/ml ^{3}H-G6P were added to the incubation system. At the time reported in the figure 250ml incubation mixture were withdrawn, diluted into 4 ml of 250mM 'ice-cold' sucrose and rapidly filtered onto Whatman GF/C filters under vacuum [16]. The radioactivity retained onto the filters was measured by beta-scintillation counting in a Beckman LS-5100 counter.

Mg^{2+} Determination

Following attainment of plasma membrane (digitonin) or endoplasmic reticulum (taurocholate) permeabilization, aliquots of the cell mixture (200–250μg protein/ml) were withdrawn and sedimented in microfuge tubes (7,000 rpm × 4 min). Supernatants were removed and assessed for Mg^{2+} content by atomic absorbance spectrophotometry (AAS) in a Perkin-Elmer 3100. The cell pellets were resuspended in 500μl 10% HNO3, sonicated by 2 pulses of 10 sec each at 15 sec intervals on ice with a Brandson sonicator at setting 7, and digested overnight. Following sedimentation of denaturated protein, Mg^{2+} content in the acid extract was measured by AAS upon appropriate dilution. Contaminant Mg^{2+} present in the cytosol-like incubation buffer was also measured by AAS and found to range between 2 to 5μM.

Protein Determination

Protein concentrations were assayed by the method of Bradford [22].

Statistical Analysis

Data were analyzed by pared t-test with a significance of $p<0.05$.

RESULTS

Addition of G6P to digitonin-permeabilized hepatocytes results in a time- and dose-dependent hydrolysis of G6P and generation of glucose and inorganic phosphate (Pi) into the incubation system [16]. In the present study, G6P concentrations ranging from 1 to 10mM were added to digitoning-permeabilized hepatocytes, and the hydrolysis rate measured as the amount of Pi generated into the incubation system at various time points. Maximal hydrolysis was observed upon addition of 5mM G6P, not increasing significantly at higher G6P concentrations (*not shown*). Hence, this concentration of G6P was used in the majority of the experiments reported in this study.

As illustrated in Fig. 1A, varying Mg^{2+} concentrations in the incubation system had a bi-phasic effect on G6P hydrolysis. Glucose 6-phosphate hydrolysis and inorganic phosphate generation increased at an external Mg^{2+} concentration of 0.25mM as compared to 0 mM $[Mg^{2+}]o$, but decreased in the presence of larger extra-reticular Mg^{2+} concentrations (i.e. 0.5 or 1mM). In keeping with the physiological range of cytoplasmic Mg^{2+} concentration within mammalian cells, concentrations of Mg^{2+} larger than 1 mM were not tested. Radioisotope distribution of G6P indicated that over 45 min of incubation, digitonin-permeabilized hepatocytes incubated in the presence of 0.25mM Mg^{2+} accumulated 30±3% more G6P than hepatocytes incubated in the presence of nominal 0mM Mg^{2+} (4.90±0.27 vs. 3.76±0.19 μmol G6P/mg protein/45 min, respectively, n=4 for both experimental conditions, $p<0.001$) whereas hepatocytes incubated in the presence of 0.5 or 1mM Mg^{2+} accumulated ~10% more G6P (e.g. 4.19±0.21 μmol G6P/mg protein/45 min, n=4, for cells incubated in the presence of 1mM Mg^{2+}) as compared to hepatocytes incubated in the presence of nominal 0mM Mg^{2+}.

The effect on G6P hydrolysis was Mg^{2+} specific, as it was not observed when permeabilized hepatocytes were incubated in the presence of equivalent concentrations of other divalent cations (Table I). The enhanced hydrolysis in the presence of 0.25mM $[Mg^{2+}]o$ was observed at all the concentrations of G6P tested (Fig. 1B).

The biphasic effect of Mg^{2+} appears to be specific for G6P. In experiments in which 1mM PPi was used as the hydrolytic substrate instead of G6P, the amount of Pi generated increased over time in a manner directly proportional to the Mg^{2+} concentration present in the incubation system (Fig. 2). Increasing the concentration of PPi beyond 1mM did not result in a larger hydrolytic activity (*not shown*).

To confirm that the effect of Mg^{2+} on G6P and PPi was specific, EDTA was added at a selected time to chelate Mg^{2+} from the system. Experimental samples containing either nominal 0 (i.e. no Mg^{2+} added) or 1mM Mg^{2+} were split into two samples after 15 min incubation. While one sample retained the indicated Mg^{2+} concentration, the other received a concentration of EDTA calculated to decrease the level of Mg^{2+} to 0mM [21]. As shown in Fig. 3A, chelating Mg^{2+} by EDTA resulted in a decreased G6P hydrolysis rate as compared to the EDTA-free counterpart. The net change in G6P hydrolyzed, reported in the inset of Fig. 3A, indicates a 20–25% increase in hydrolysis rate in the presence of 0.25mM Mg^{2+} vs. 0 mM Mg^{2+} at 30 and 45 min. Following EDTA addition, G6P hydrolysis was marked attenuated, reaching levels similar to those observed at 0mM Mg^{2+} in the absence of EDTA (*Fig, 3A*). Similar experiments were performed using 1mM PPi as substrate, in the presence of 1mM Mg^{2+}, as PPi was maximal at this Mg^{2+} concentration. Also in this case, the addition of EDTA at a concentration sufficient to chelate Mg^{2+} level to 0mM resulted in a complete inhibition of PPi hydrolysis (Fig. 3B, and relative inset). Similar inhibition of G6P and PPi hydrolysis were obtained adding EDTA at t = 0 min after quickly splitting the sample (*not shown*).

To exclude that the effect of Mg^{2+} on G6P or PPi hydrolysis was due to a leak of Mg^{2+} across the ER membrane over time, the amount of Mg^{2+} retained within the ER of digitonin permeabilized hepatocytes was measured by AAS. The values, reported in Table 2, indicate that Mg^{2+} content did not change significantly during our incubation procedure irrespective of the absence or the presence or EDTA or the amount of Mg^{2+} added to the system.

The next series of experiments was designed to determine whether Mg^{2+} effect was at the level of G6P transport mechanism, or intra-ER Mg^{2+} concentration was involved in the process. Taurocholate was used to permeabilize the ER membrane and allow G6P to access the hydrolytic site bypassing the G6P transport mechanism. Following addition of taurocholate a loss of Mg^{2+} from the ER lumen larger than that observed in digitonin-only treated cells was observed (Table 2). Under these conditions, the effect of 0.25mM extra-reticular Mg^{2+} on G6P hydrolysis was completely abolished (Fig. 4A). Similar results were obtained when 5mM mannose 6-phosphate (M6P) instead of G6P (Fig. 4B) were used as alternative substrates.

Histone 2A [19] and alamethicin [9,19] have been used in alternative to taurocholate or deoxycholate to enhance permeability of ER membrane to G6P bypassing the transport mechanism. The effect of either of these agents on G6P hydrolysis is reported in Fig. 5. As the figure illustrates, in the presence of alamethicin (3μg/ml) or histone 2A (250μg/ml or isodose vs. the protein content in the incubation system, [9,19]) G6P hydrolysis in the presence of 0.25mM extra-reticular Mg^{2+} was significantly lower than that observed in cells permeabilized only with digitonin. Following the addition of alamethicin or histone 2A, a similar amount of Mg^{2+} was lost from the ER (Table 2). No additional Mg^{2+} loss was observed when concentrations of histone 2A or alamethicin larger than those indicated above were used. When the Mg^{2+} ionophore A23187 was used to decrease intra-luminal Mg^{2+} concentration [20] without altering ER permeability to G6P, the amount of Mg^{2+} retained within the hepatocyte was comparable to that measured following administration of histone 2A or alamethicin (Table 2). Under these conditions, G6P hydrolysis was only partially stimulated (plus 13%) by the addition of 0.25mM extra-reticular Mg^{2+} as compared to the hydrolysis occurring in the presence of 0mM Mg^{2+} (Fig. 5). This trend did not reach statistical significance and was further abolished in the presence of EDTA to levels similar to those observed with alamethicin or histone 2A (Fig. 5).

Perturbation of endo-luminal Mg^{2+} content by taurocholate, alamethicin or A23187 also abolished the effect of extra-reticular Mg^{2+} (1mM) on PPi hydrolysis (*not shown*).

DISCUSSION

Cytoplasm and endoplasmic reticulum (ER) represents two of the main Mg^{2+} compartments within the hepatocyte [1]. While in the cytoplasm Mg^{2+} is predominantly in the form of a complex with ATP and other phosphonucleotides [2], the presence of a total Mg^{2+} concentration in the order of 16 to 20mM, at least in the rough component of the ER [1], has questioned the physiological role the cation may play within the ER compartment. Protein synthesis is a main area in which ER Mg^{2+} appears to play a major regulatory role [23]. Cytoplasmic and/or ER Mg^{2+} concentration have also been implicated in regulating the amplitude of Ca^{2+} release from the endoplasmic [24] or sarcoplasmic reticulum [25] via IP_3 and ryanodine receptor, respectively. These conditions, however, do not exclude the possibility that reticular and/or trans-reticular Mg^{2+} gradient is involved in regulating other ER functions.

Glucose 6-phosphatase is one of the main enzymes operating within the endoplasmic reticulum of the hepatocyte. Although two different models have been proposed for its operation [12, 13], consensus is there that G6P, the substrate of choice, has not free access to the hydrolytic site of the G6Pase but has to be transported via a specific transport mechanism. Following hydrolysis, glucose and Pi are released into the cytoplasm via two distinct transport mechanisms separated from that utilized by G6P [13]. Aside from some organic compounds [26], no specific inhibitory or regulatory agents for G6P transport and/or hydrolysis have been reported. Hence, it has been proposed that the G6P hydrolysis rate is regulated by the rate of substrate delivery to the hydrolytic site [12,13].

The present study was undertaken to test the hypothesis that cytoplasmic Mg^{2+} or endoplasmic reticulum Mg^{2+} concentration could play a role in regulating G6P hydrolysis within the hepatocyte.

Cytoplasmic Mg^{2+}

Varying the concentrations of extra-reticular Mg^{2+} results in a biphasic effect on G6P hydrolysis rate (Fig. 1, and radioisotope distribution). Hydrolysis is larger at a concentration of 0.25mM Mg^{2+} and decreases at higher Mg^{2+} concentrations, being the lowest at ~1mM Mg^{2+}. This effect is specific for Mg^{2+}, is abolished by EDTA addition, and is observed at all the concentrations of G6P tested. The possibility this effect is due to a leak of Mg^{2+} from the ER is not supported by two observations. First, the amount of Mg^{2+} retained within the ER does not vary significantly over the time course of our incubation irrespective of the extra-reticular Mg^{2+} concentration utilized (Table 2). Second, the addition of EDTA at a dose calculated to reduce the extra-reticular Mg^{2+} content to 0mM (Fig. 3) reduces the hydrolysis rate while minimally altering the amount of Mg^{2+} retained within the ER. If dependent on a non-specific leak from the ER luminal reservoir, the Mg^{2+} effect should continue to persist in the presence of EDTA since the amount of EDTA added (i.e. ~0.35mM for a 0.25mM Mg^{2+} concentration) would be insufficient to chelate all the Mg^{2+} present within the ER [1] in addition to that added to the incubation system. Furthermore, by acting as 'Mg^{2+}-sink', EDTA should amplify the leak of Mg^{2+} from the ER lumen. The inhibitory effect of EDTA also excludes that the decrease in hydrolysis rate observed at higher Mg^{2+} concentrations is due to a 'chelating' effect of Mg^{2+} on G6P. If this was indeed the case, by removing Mg^{2+} from its G6P binding, EDTA addition should result in an increase rather than a decrease in hydrolysis. Although the modalities by which Mg^{2+} redistributes across the ER membrane are not elucidated, it is unlikely that the addition of 0.25mM Mg^{2+} to the extra-reticular space (cytoplasm) changes intra-luminal Mg^{2+} concentration (16 to 18mM, [1]) to a significant extent able to influence the activity of G6Pase hydrolytic site. On the other hand, EDTA appears to have a marked inhibitory effect in samples containing a nominal 0mM Mg^{2+} (i.e. no Mg^{2+} added). This is likely to depend on a chelating effect of EDTA on Mg^{2+} present as contaminant in the buffer (2–5μM), or as carry-over from the cell suspension following permeabilization

by digitonin. The possibility that EDTA is chelating Mg^{2+} bound to phospholipids and/or proteins of the ER membrane, however, cannot be excluded altogether. Lastly, G6Pase activity appears to be qualitatively and quantitatively different in the rough and smooth portion of the ER, at least in microsomal vesicles [7]. Because the present study was carried out in permeabilized cells, our results do not clarify whether the effect of Mg^{2+} predominates in the smooth compartment, in which G6Pase is reported to be more active [7], or occurs instead on both rough and smooth portions of the ER.

Endo-luminal Mg^{2+}

The stimulatory effect of Mg^{2+} on G6P does not take place in digitonin-permeabilized hepatocytes treated with taurocholate (Fig. 4), histone 2A or alamethicin (Fig. 5). All these agents induce a major Mg^{2+} loss (50% or more) from the ER (Table 2) that is not significantly affected by the concentration of extra-reticular Mg^{2+} (0mM or 0.25mM) present in the incubation system (Table 2). All these agents, however, increase the delivery of G6P to the hydrolytic site of the G6Pase bypassing the transport mechanism. This explains why the level of Pi generated under these experimental conditions is larger than that generated in hepatocytes permeabilized only with digitonin. A lack of Mg^{2+} effect is also observed on the alternative hydrolysis substrate M6P.

Perturbing ER Mg^{2+} content with the ionophore A23187, which does not alter G6P delivery to the ER lumen, however, results in a partially maintained stimulatory effect of extra-reticular Mg^{2+} on G6P hydrolysis, an effect that is completely abolished by EDTA addition (Fig. 5). Although not statistically significant when compared to the hydrolysis in the absence of ionophore, this effect 1) supports the idea that extra-reticular Mg^{2+} acts at the level of the G6P transport mechanism, and 2) suggests a modulating role of trans-reticular Mg^{2+} gradient on G6P transport and/or hydrolysis. Since the ionophore A23187 moves Mg^{2+} down its concentration gradient in exchange for two protons, it could be argued that G6P hydrolysis rate is affected by intra-reticular pH acidification occurring under these experimental conditions. While not fully consistent with the notion that pH 6.8 is optimal for G6Pase hydrolysis [13], the possibility that intra-reticular pH drops below the optimal range for hydrolytic activity cannot be completely dismissed.

Effect of Mg^{2+} on PPi transport and hydrolysis

Changes in cytoplasmic and intra-reticular Mg^{2+} level appear to have a significant regulatory role on PPi hydrolysis as well. The stimulatory effect of Mg^{2+} on the PPi transporter, however, is different from that observed for G6P, in that it is dose-dependent and not biphasic, and it is completely abolished by removal of Mg^{2+} following EDTA addition. This observation strongly suggests that also in this case Mg^{2+} plays a regulatory role at the level of PPi transport rather than its hydrolysis.

Patho-physiological relevance

The biphasic effect of cytoplasmic Mg^{2+}on T1 transporter and G6P hydrolysis is interesting. Yet, the stimulatory effect occurs at a concentration that is not physiological for the hepatocyte. As attested by ^{31}P-NMR [3] or fluorescence indicator [27] measurements, cytoplasmic Mg^{2+} level ranges between 0.5–0.7 mM under basal, un-stimulated conditions. Total and free Mg^{2+} concentrations, however, decrease significantly under diabetic conditions [20,28] or prolonged exposure to ethanol [29], reaching values close to the concentration of 0.25mM tested here. Diabetic conditions are indeed characterized by an increased G6Pase activity [30], a phenomenon that has been explained by the lack of repression on G6Pase gene due to insulin absence (type-I diabetes) or reduced effectiveness (type-II diabetes). Because type-I and type-II diabetes are both characterized by a decrease in cellular and cytoplasmic Mg^{2+} [20], it is tempting to speculate that the enhanced activity of G6Pase observed under diabetic

conditions is due to a genomic effect, linked to insulin deficiency, and in part to a 'stimulatory' effect of a reduced cytoplasmic Mg^{2+} concentration on T1 transporter. Further studies in hepatocytes from diabetic animals are necessary to confirm if this is indeed the case.

Conclusions

Taken together, our results suggest that cytoplasmic Mg^{2+} level plays a significant role in regulating G6P and PPi transport and hydrolysis in liver cells. This effect appears to be at the level of the transport mechanisms for PPi and G6P, or surrounding phospholipid environment, rather than on the hydrolytic site of G6Pase enzymatic complex. In the case of G6P, our results are consistent with either G6Pase model proposed, and do not necessarily support one model over the other. In the case of the *substrate-transport model*, Mg^{2+} effect adds a new level of complexity and regulation at the level of T1, which already represents the rate-limiting factor for the G6Pase system. In the *conformational flexibility substrate-transport model*, instead, Mg^{2+} may regulate the conformational changes of the hydrophilic region, located at the cytosolic side, which occurs upon substrate binding and is responsible for the delivery to the intra-luminal catalytic site. As indicated previously, it is possible that intra-reticular Mg^{2+} content provides an additional level of modulation on G6P transport and hydrolysis. This effect of Mg^{2+} is likely to have a major impact under diabetic conditions rather than under physiological conditions.

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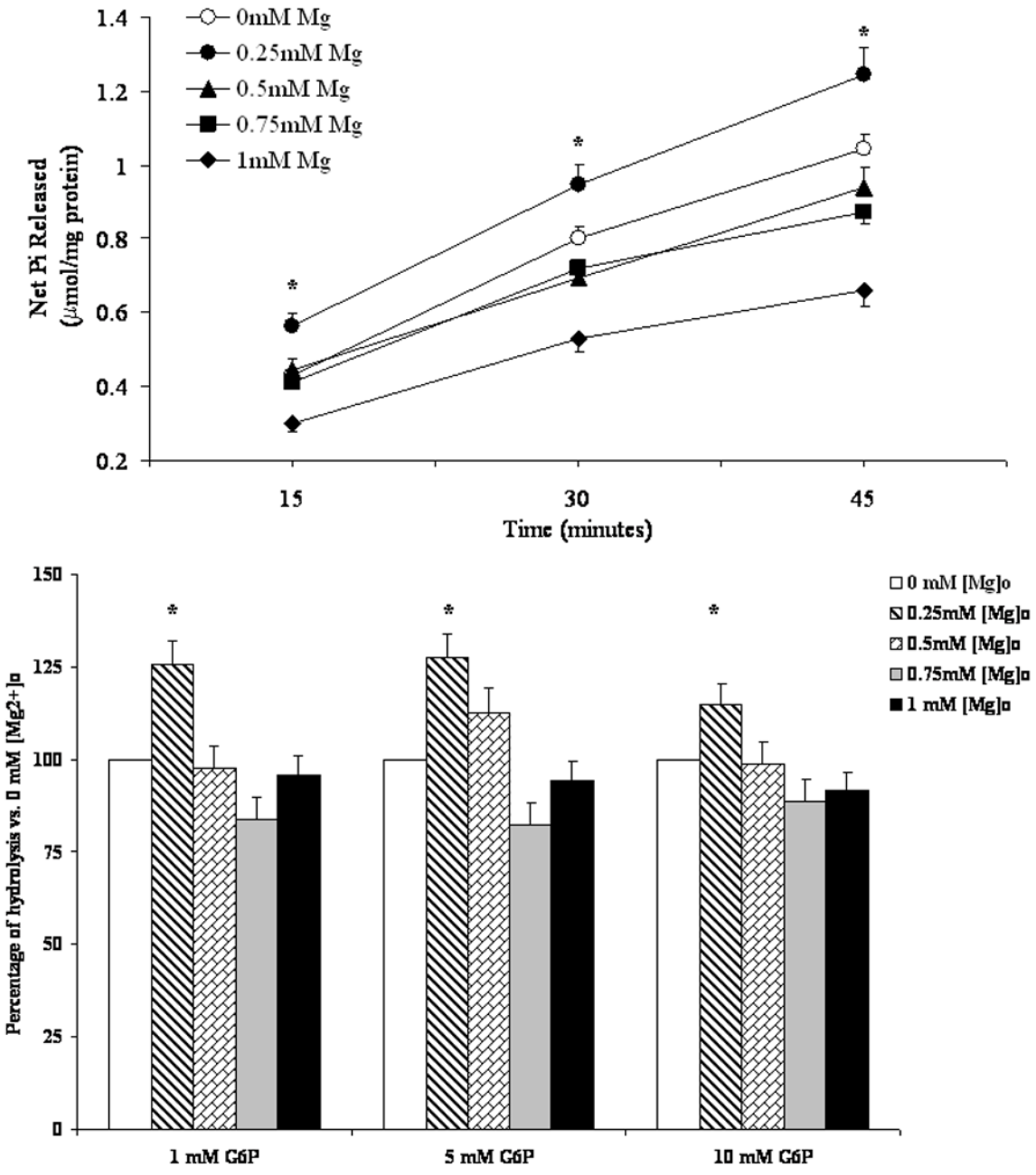

Figure 1. G6P hydrolysis in the presence of varying Mg^{2+} (Fig. 1A) and G6P (Fig. 1B) concentrations

Time course of net G6P hydrolysis in digitonin-permeabilized hepatocytes incubated in the presence of 5mM G6P and varying Mg^{2+} concentrations, expressed as μmol Pi/mg protein, is reported in Fig. 1A. Hydrolysis in the presence of 1mM, 5mM, and 10mM G6P and varying Mg^{2+} concentrations at time 45 min is reported in Fig 1B as percent change vs. hydrolysis in the presence of 0mM external Mg^{2+}. Data are means±S.E. of 6 experiments for each condition, each performed in duplicate. *Statistically significant vs. other samples.

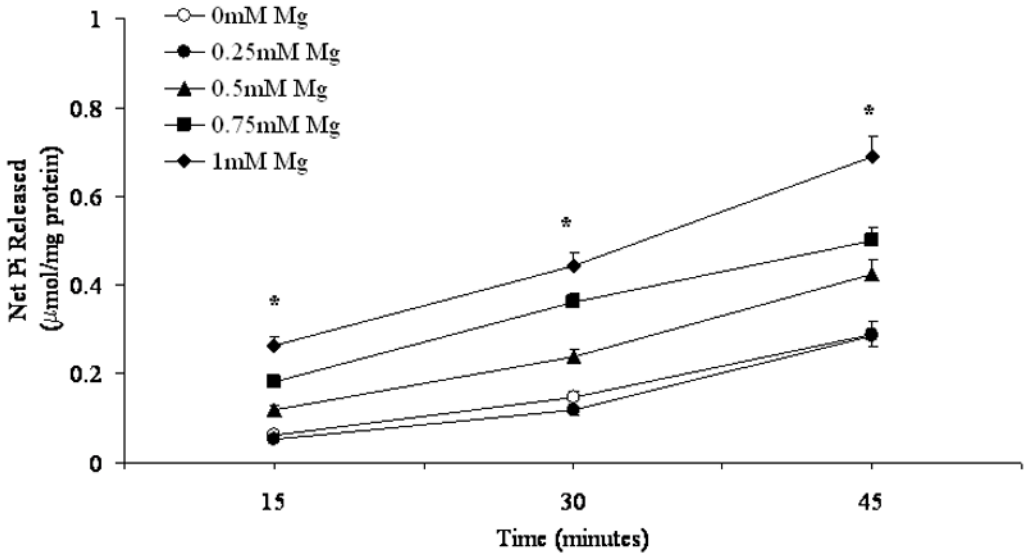

Figure 2. PPi hydrolysis in the presence of varying Mg^{2+} concentrations

Time course of net PPi hydrolysis in digitonin-permeabilized hepatocytes incubated in the presence of 1mM PPi and varying Mg^{2+} concentrations is reported. Data, expressed as μmol Pi/mg protein, are means±S.E. of 6 experiments for each condition, each performed in duplicate. *Statistically significant vs. other samples. All t=15 min, t=30 min and t=45 min of samples incubated in the presence of 0.5mM, 0.75mM and 1mM Mg^{2+} are statistically significant vs. the corresponding time points of 0mM and 0.25mM Mg^{2+} samples. Labeling is omitted for simplicity.

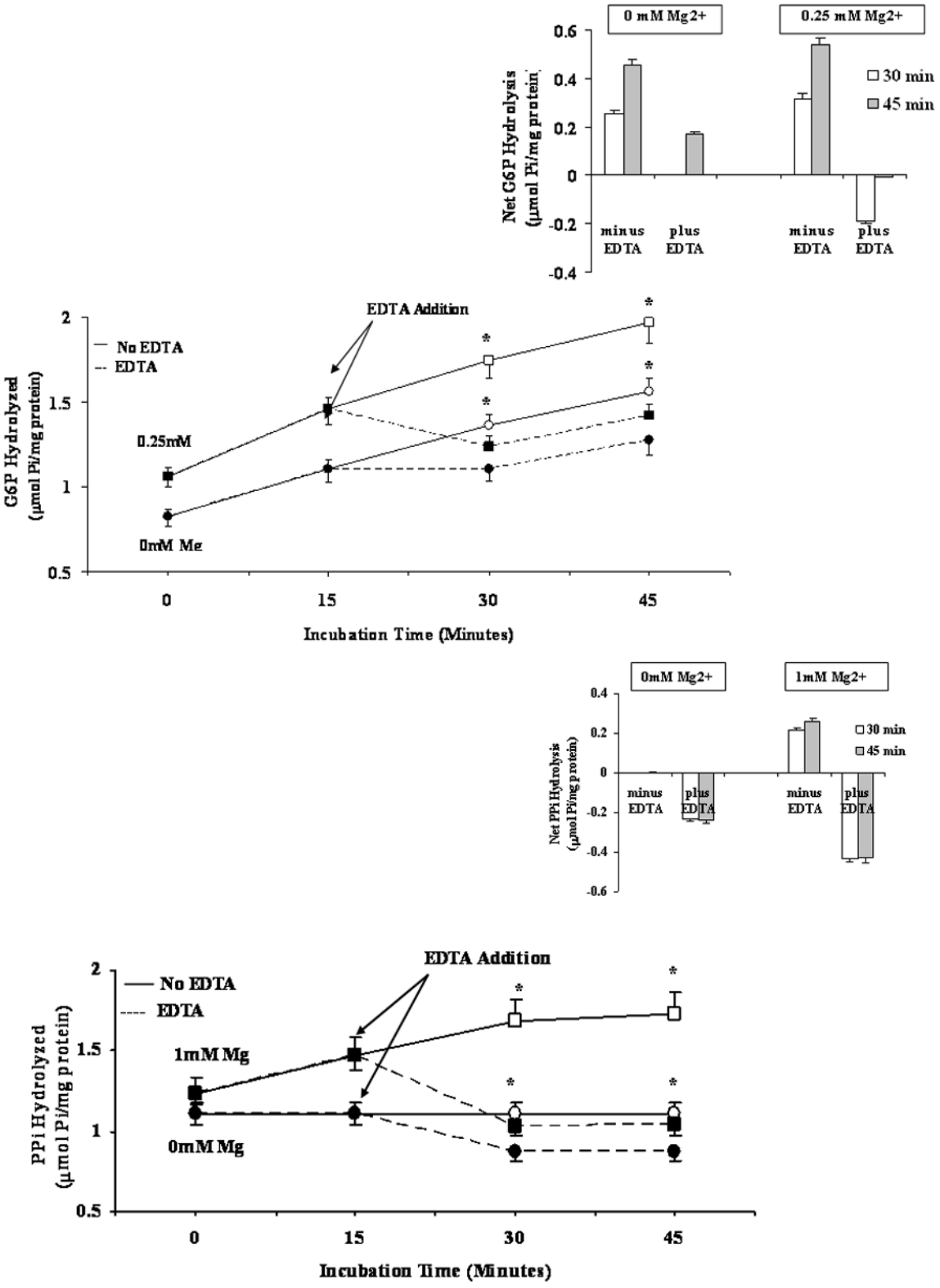

Figure 3. G6P (Fig. 3A) and PPi (Fig. 3B) hydrolysis in the presence of varying Mg^{2+} concentration, in the absence or in the presence of EDTA

Digitonin-permeabilized hepatocytes were incubated in the presence of 5mM G6P (Fig. 3A) and 1mM PPi (Fig. 3B) in a medium containing either nominal 0mM or 0.25mM Mg^{2+} (Fig, 3A), or 0mM and 1mM Mg^{2+} (Fig. 3B). After 15 min incubation, the samples were split into two, one of which received a concentration of EDTA sufficient to chelate all external Mg^{2+} [20]. The inset in each figure reports the net change in hydrolysis at time=30min and time=45 min in the absence and in the presence of EDTA vs. time=15min. Data are means±S.E. of 5 different preparations. *Statistically significant vs. the corresponding sample in the presence of EDTA. Labeling in the inset is omitted for simplicity.

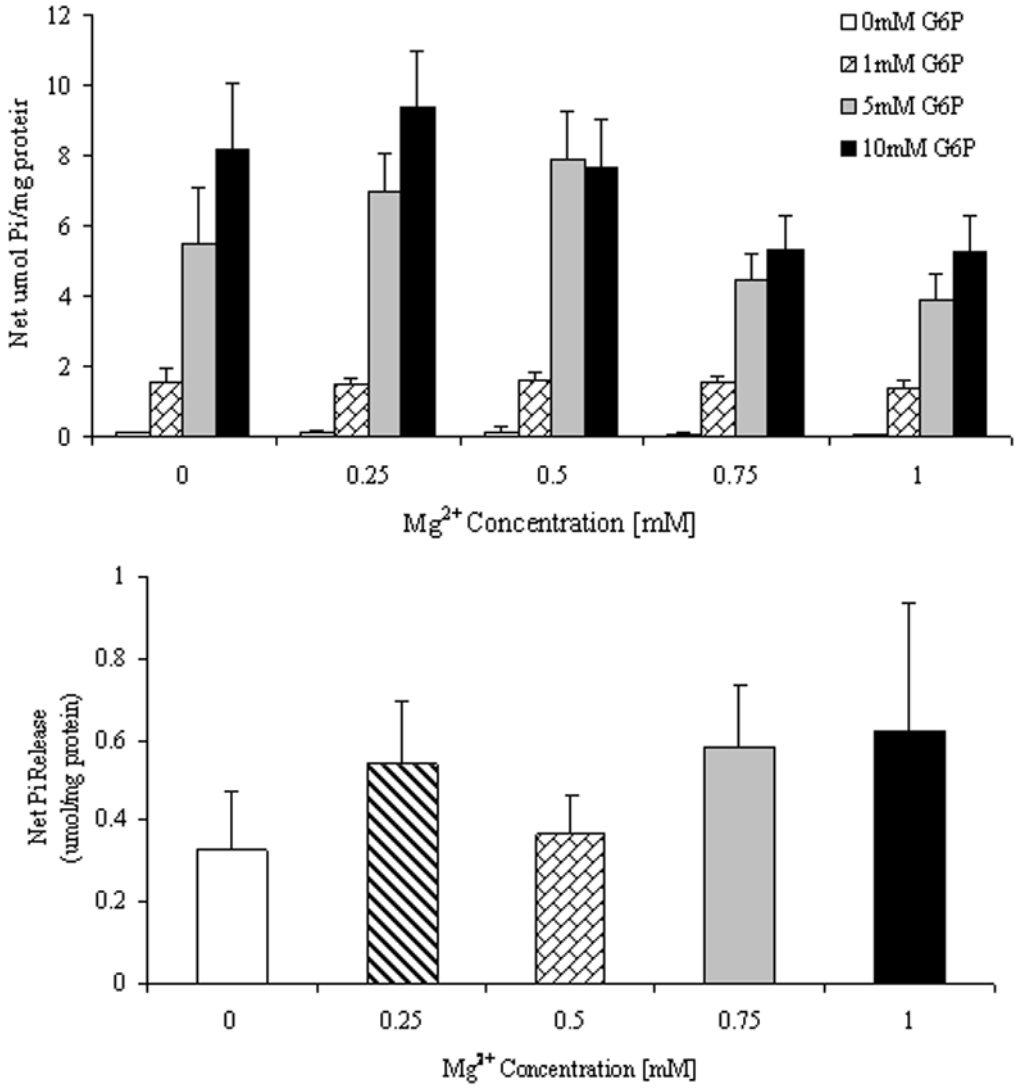

Figure 4. G6P (Fig. 4A) and M6P (Fig. 4) hydrolysis in the presence of varying Mg^{2+} concentrations

Digitonin- and taurocholate-permeabilized hepatocytes were incubated in the presence of varying Mg^{2+} and G6P concentrations (Fig. 4A) or 5mM mannose 6-phosphate (M6P, Fig. 4B). Net hydrolysis at time = 45 min is reported for all samples. Data are means±S.E. of 6 experiments for each condition, each performed in duplicate.

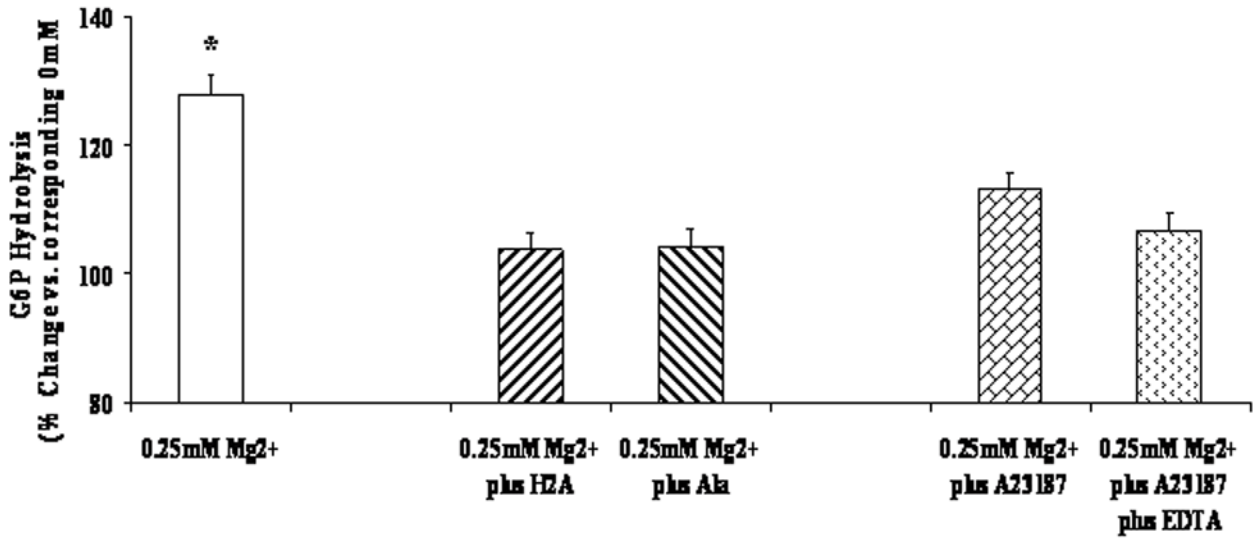

Figure 5. G6P hydrolysis in the presence of varying histone 2A or alamethicin

Digitonin-permeabilized hepatocytes were incubated in the presence of 5mM G6P, 0.25mM Mg^{2+}, and 250μg/ml histone 2A or 3μg/ml alamethicin or 2μg/m A23187 (plus or minus EDTA). Net hydrolysis at time = 45 min is reported as percent variation vs. the corresponding sample incubated in the presence of 0mM Mg^{2+}. Data are means+S.E. of 4 experiments for each condition, each performed in duplicate. *Statistically significant vs. other samples.

Table 1

Effect of Different Cations on G6P hydrolysis

		Net G6P hydrolysis 15 min	30 min	Percent change vs. 1mM 15 min	30 min
Mg^{2+}	0.25mM	1.14±0.2*	1.90±0.1*	121.8	128.7
	1mM	0.94+0.1	1.48+0.2		
Mn^{2+}	0.25mM	1.32+0.2	1.82+0.3	105.6	104.0
	1 mM	1.25+0.4	1.75+0.2		
Ca^{2+}	0.25mM	1.14+0.3	1.93+0.2	108.6	109.7
	1mM	1.05+0.4	1.76+0.5		
Co^{2+}	0.25mM	1.30+0.2	1.92+0.3	103.2	105.5
	1mM	1.26+0.4	1.82+0.2		
Ni^{2+}	0.25mM	1.25+0.3	2.00+0.4	104.2	96.6
	1mM	1.20+0.2	2.07+0.5		

G6P (5 mM) was added at time = 0 min. Net hydrolysis was calculated vs. the hydrolysis at time = 0 min, and expressed as μmol Pi/mg protein. Hydrolysis at time = 45 min was omitted for simplicity. Data are means +S.E. of 4 different preparations.

*Statistically significant vs. corresponding values at 1mM.

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Table 2

Changes in the amount of Mg^{2+} retained within the hepatocytes following addition of digitonin, EDTA, taurocholate, histone 2A, alamethicin, or A23187.

	Mg^{2+} content		Percent Variation at 45 min vs. no Agent
	0 min	45 min	
Digitonin-Permeabilized			
0mM Mg^{2+}/no EDTA	21.4+1.8	20.8±2.3	
0mM Mg^{2+}/EDTA		20.0±1.2	96.2
0.25mM Mg^{2+}/no EDTA	22.2±0.9	22.0±1.5	
0.25mM Mg^{2+}/EDTA		20.9±1.9	95.0
0mM Mg^{2+}/no H2A	22.1±1.7	21.5±1.1	
0mM Mg^{2+}/H2A		10.5±1.1*	48.8
0.25mM Mg^{2+}/no H2A	21.3.1±1.5	21.9±1.5	
0.25mM Mg^{2+}/H2A		11.4±1.3*	52.1
0mM Mg^{2+}/no Ala	22.6±0.8	22.3±2.5	
0mM Mg^{2+}/Ala		10.9±1.3*	48.9
0.25mM Mg^{2+}/no Ala	21.2±1.3	21.0±2.4	
0.25mM Mg^{2+}/Ala		11.6±1.2*	55.2
0mM Mg^{2+}/no A23187	21.1±1.0	22.1±2.2	
0mM Mg^{2+}/A23187		11.7±1.5*	52.9
0.25mM Mg^{2+}/no A23187	21.8±1.2	22.6±1.4	
0.25mM Mg^{2+}/A23187		10.0±2.0*	44.2
0.25mM Mg^{2+}/A23187/EDTA		9.76±2.7*	43.2

Values (nmol Mg^{2+}/mg protein) represent total cellular Mg^{2+} content at the indicated time points. Only 1 value for time = 0 min is reported as samples were split at time = 15 min to continue the incubation in the absence or in the presence of EDTA (~0.3mM)., histone 2A (250μg/ml), alamethicin (3μg/ml), or A23187 (2μg/ml). Data are means+S.E. of 4 different preparations, each performed in duplicate.

* Statistically significant vs. corresponding value ($p<0.001$).