Insulin and glucose mediate opposite intracellular ionized magnesium variations in human lymphocytes

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Abstract

Insulin is capable of increasing intracellular magnesium, although very little is known about the effect of insulin on the biologically active fraction of magnesium, i.e. the ionized quota (Mg_i^{2+}), its interactions with glucose, and the cellular mechanisms involved in these processes. We studied the interactions of the effects of insulin and glucose on intracellular ionized magnesium in human lymphocytes. Mg_i^{2+} was measured using a fluorimetric method and the Mg^{2+} -sensitive dye, fura-2. We found that insulin significantly increases the Mg_i^{2+} (without insulin $227 \pm 14 \mu M$, with $10 \mu U/ml$, insulin $301 \pm 30 \mu M$, $P < 0.0001$, $n = 12$) in a dose-dependent manner in all three glucose concentrations tested (5, 7 and 15 mmol/l). The half-maximal effect of insulin was

approximately $0.8 \mu U/ml$. Glucose and insulin showed opposite effects in their ability to modify Mg_i^{2+} in lymphocytes. Inhibitors of the membrane $Na^+ - Mg^{2+}$ transport system and of phosphatidylinositol (PI) 3-kinase abolish the insulin-mediated increase of Mg_i^{2+} , thus suggesting that insulin is capable of increasing Mg_i^{2+} by modulating the activity of this transport system, possibly through the mediation of PI 3-kinase activation. Taking into account the relationship between insulin and glucose plasma levels and their opposing effects on Mg_i^{2+} , this mechanism may represent the two limbs of a biphasic regulatory system of Mg_i^{2+} in both physiological and pathological conditions.

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Introduction

A potential role of magnesium deficiency in the pathogenesis of type 2 diabetes mellitus has been recently proposed based on epidemiological, nutritional, and experimental data. In fact, large observational studies have described an association between low serum magnesium levels and type 2 diabetes (Ma *et al.* 1995). Accordingly, magnesium supplementation might be useful in the prevention and treatment of diabetes (Schnack *et al.* 1992). *In vitro* studies have shown an effect of magnesium on insulin secretion by the pancreas and on the mechanism of peripheral insulin resistance (Hwang *et al.* 1993). Our group has shown that intracellular ionized magnesium (Mg_i^{2+}) concentrations are decreased in patients with insulin resistance syndrome (Delva *et al.* 1996, 1998a) and that peripheral insulin resistance is related to Mg_i^{2+} levels (Delva *et al.* 1998b). Furthermore, we have shown *in vitro* that high extracellular glucose at concentrations compatible with those found in diabetes can reduce the concentration of Mg_i^{2+} (Delva *et al.* 2002). Insulin has been shown to increase the Mg_i^{2+} concentration in human erythrocytes (Barbagallo *et al.* 1993, 1997), platelets (Hwang *et al.* 1993), and the total intracellular magnesium in rat ventricular myocytes (Romani *et al.* 2000), and prevents Mg^{2+} extrusion induced by isoproterenol (Romani *et al.* 2000). Nonetheless, very little is

known about the effect of insulin on the biologically active fraction of magnesium, i.e. the ionized quota, in nucleated human-derived cells, its interactions with glucose, and the cellular mechanisms involved. We have studied the interactions between insulin and glucose on Mg_i^{2+} in human lymphocytes in order to shed light on the cellular mechanisms involved in these processes.

Material and Methods

Reagents

The Ficoll-Histopaque reagent was obtained from Sigma Chemical, as were the RPMI 1640, BSA free fatty acids, and all the sugars utilized. $MgCl_2$, lanthanum oxide (ultrapure grade), wortmannin, vandate and insulin were obtained from Sigma. Fura-2-acetoxymethyl ester and fura-2 acetoxymethyl ester were provided by Molecular Probes Inc. (Eugene, OR, USA).

Preparations of human lymphocytes

All studies were performed on human lymphocytes obtained from the local blood bank from healthy blood donors. Consent was obtained from the subjects after the nature of the

procedure was explained. Peripheral blood lymphocytes were isolated as follows: total blood was diluted with RPMI 1640 medium (HEPES modification, glucose-free and supplemented with MgSO_4 to reach the concentration of 0.8 mM) and layered carefully onto Histopaque 1077 and then centrifuged for 30 min at 400 g. The layer of lymphocytes was carefully aspirated and washed twice in RPMI 1640 for 10 min at 150 g. The cells thus obtained were allowed to sediment for 30 min in culture flasks. The supernatant was then transferred into tubes and centrifuged, and the lymphocytes thus obtained were resuspended in the same medium. The percentage of lymphocytes always exceeded 95% and the vitality assessed as Trypan Blue exclusion was always higher than 97%.

Measurement of free intralymphocyte magnesium

We used the method previously described by Delva *et al.* (1996). Lymphocytes were counted by means of a Coulter Counter (Coulter Electronics Ltd, Dunstable, Beds, UK). The cells were washed three times with RPMI 1640 and then three separate aliquots of lymphocytes (6×10^6 cells each) were suspended in RPMI 1640 with insulin (or the relevant substance) and three aliquots (6×10^6 cells each) without insulin (control cells). In some experiments, when the concentration of extracellular Mg^{2+} was varied, RPMI 1640 was substituted with Bulher solution containing (in mM) NaCl 145, KCl 5, Na_2HPO_4 0.5, HEPES 10, CaCl_2 1, MgSO_4 0.3. All the aliquots had BSA 0.1% (v/v) and the cell permeant fura-2-acetoxymethyl ester 10 μM added to them for 1 h at 37 °C. After centrifugation, the cells were washed twice in the same medium to remove extracellular dye and resuspended in the same medium at room temperature for 45 min for complete deesterification of the dye. For the measurement of intracellular Mg^{2+} , the buffer utilized contained (in mM) 140 NaCl, 5 KCl, 1.8 CaCl_2 , 0.8 MgSO_4 , and 15 HEPES with or without insulin for the insulin-incubated aliquots and without insulin for the insulin-free aliquots (pH 7.4 at 31 °C). After washing off the 1640 medium, the cells were added to the above pre-warmed medium (31 °C) just before the fluorimetric measurements were performed. Fluorescence emission at 510 nm (slit width 10 nm) was measured with alternate excitation at 335 and 370 nm (slit width 10 nm) within a thermostatically controlled cuvette holder (31 °C) in a Hitachi F-2000 fluorescence spectrophotometer. Autofluorescence contributed to less than 1% of the total fluorescence values. One centimeter quartz cuvettes were used for all experiments. After reading the initial fluorescence emission resulting from excitation at 335 and 370 nm, 5 mM EDTA and 5 mM EGTA were added to the cuvette. Since extracellular Mg^{2+} and Ca^{2+} were chelated in the medium, a rapid-step change in fluorescence at both wavelengths occurred due to dye leaking from inside the cells. The immediate (<10 s) change in fluorescence intensities at

both wavelengths after the addition of EDTA and EGTA was considered in order to calculate the free resting intracellular Mg^{2+} . Triton X-100 was then added to a final concentration of 0.1% (v/v) to lyse the cells, and since the cells were in a medium containing EDTA and EGTA, it was possible to determine the minimum fluorescence ratio, $R_{\min}$. Subsequently, MgSO_4 (100 mM) was added to obtain the maximum fluorescence ratio, $R_{\max}$. Intracellular-free Mg was measured in triplicate and was calculated according to (Raju *et al.* 1989). The K_d of intracellularly generated fura-2 for Mg^{2+} in our experimental condition was calculated to be 2.1 mM as previously described (Delva *et al.* 2004).

Measurement of free intralymphocyte magnesium in the cytosol and intracellular organelles

We performed an indirect estimate of Mg^{2+} in the cytosol and intracellular organelles of lymphocytes, both in the presence of insulin and in its absence, by differential permeabilization of the cells (Delva *et al.* 2004). We used the procedure described above to measure lymphocyte steady-state Mg^{2+} further by adding, after the addition of EGTA/EDTA mixture, digitonin (25 mg/l) in order to permeabilize the cell plasma membrane. In this condition, the cytosolic free magnesium is expected to leave the cell following its outwardly directed gradient and all the fluorescence measured is derived from intracellular organelles. Then, we indirectly calculated the cytosolic free magnesium concentration by subtracting the magnesium-free concentration in the organelles from the lymphocyte steady-state Mg^{2+} concentration of the cell.

Measurement of free intralymphocyte calcium

The intracellular Ca^{2+} of lymphocytes was measured using fura-2 as previously described by Delva *et al.* (1996). Six aliquots of cells (6×10^6 each) were incubated following the same strategy used for Mg^{2+} determinations (with or without insulin) with 5 μM fura-2-acetoxymethyl ester for 1 h at 37 °C, and then washed twice and left for 45 min at room temperature before any measurements were made. For fluorimetric measurements, the same buffer used for Mg^{2+} assay was utilized and the cells were added to a pre-warmed medium with a technique similar to that used for the Mg^{2+} assay. EGTA (10 mM) was added to chelate Ca^{2+} in the medium, thus producing a desaturation of fura-2, which leaked from inside the cells, and giving a rapid-step change in fluorescence emission at 510 nm with excitation set at both 340 and 380 nm wavelengths. This provided the ratio values for the intracellular Ca^{2+} resting calculation (Gryniewicz *et al.* 1985) using a K_d value for 175 nM Ca^{2+} /fura-2 complex as previously calculated in our experimental conditions (Delva *et al.* 2004).

The intra- and inter-assay variability (coefficient of variation) determinations were 5.8 and 3.9% for ionized Mg_i^{2+} and 4.6 and 12.7% for ionized Ca_i^{2+} determinations.

Measurement of total intralymphocyte magnesium

We utilized the method described by Elin and Hosseine (1985) with some modifications as previously described (Delva *et al.* 2004).

Measurement of intralymphocyte ATP

ATP content of human lymphocytes was measured as described by Nieminen *et al.* (1990) with minor variations (Delva *et al.* 2004).

Statistical analysis

Results are expressed as means $\pm$ S.D. if no other method is specified. Comparison between groups was performed using Student's *t*-test and considered statistically significant when the probability of the null hypothesis was below at least 5%. Confidence limits for differences in means were also provided. For multiple comparisons, a one-way ANOVA test with Bonferroni's correction for multiple comparisons was used.

Results

Effect of insulin on the intralymphocyte ionized magnesium concentration

The steady-state Mg_i^{2+} in the incubation media without insulin was $227 \pm 14 \mu M$ ($n = 12$). Incubation of lymphocytes for 2.5 h with 10 $\mu U/ml$ insulin significantly increased this value ($301 \pm 30 \mu M$, $P < 0.0001$, 95% confidence interval: 49–96 μM , $n = 12$) (Fig. 1C). Dose–response curves of Mg_i^{2+} to insulin at three extracellular glucose concentrations (5, 7, and 15 mmol/l) are shown in Fig. 1A. The effect of insulin on Mg_i^{2+} appears to be dose-dependent in all three extracellular glucose concentrations tested, with a half-maximal effect (EC_{50}) of approximately 0.8 $\mu U/ml$ insulin. Three statistically different maximal Mg_i^{2+} values were found (glucose: 5 mmol/l, 277 ± 38 ; 7 mmol/l, 231 ± 40 ; 15 mmol/l, 204 ± 35 ; ANOVA: $F = 3.5$, $P < 0.01$, $n = 6$). In Fig. 1B, the time course of the increase of Mg_i^{2+} by 10 $\mu U/ml$ insulin is measurable after 30 min, reaching a maximal effect after 120 min. The *in vitro* effects of insulin comparisons to IGF-1 and insulin-mimetic stimuli, glucose analogs, inhibitors of insulin signaling and inhibitors of glucose metabolism are summarized in Table 1. The interrelationships between the *in vitro* effects of insulin and inhibitors or regulators of transmembrane Mg^{2+} fluxes in human lymphocytes are summarized in Table 2.

Figure 1 (A) Dose–response curves of Mg_i^{2+} to insulin at three extracellular glucose concentrations (5, 7 and 15 mmol/l). The half-maximal effect (EC_{50}) for the three curves is approximately equal (0.15 $\mu U/ml$ insulin) and three statistically different maximal Mg_i^{2+} values were found. One preparation of lymphocytes was exposed to one concentration of glucose and five different concentrations of insulin. The lymphocytes used for these experiments belong to a single donor. (B) Time course of the insulin-induced (10 $\mu U/ml$) Mg_i^{2+} increase in human lymphocytes. (C) Bar graph showing the *in vitro* effect of 10 $\mu U/ml$ insulin on Mg_i^{2+} in human lymphocytes in the absence and presence of 5 mmol/l glucose. Each point is the mean $\pm$ S.D. obtained using six different preparations of lymphocytes.

Effects of differential permeabilization of lymphocytes on insulin-induced Mg_i^{2+} increases

In the absence of insulin, the lymphocyte whole-cell Mg_i^{2+} , the digitonin-treated lymphocyte Mg_i^{2+} (compartmentalized Mg_i^{2+}) and the lymphocyte cytosolic Mg_i^{2+} were 126 ± 23 , 20 ± 7 , $106 \pm 23 \mu M$, respectively ($n = 5$). In the presence of 10 $\mu U/ml$ insulin, these values were 177 ± 26 , 29 ± 4 , and

Table 1 Effect of different *in vitro* treatments on the insulin-induced Mg_i^{2+} increase in human lymphocytes

Treatment	<i>n</i>	Intralymphocyte Mg_i^{2+} concentration (μM)	<i>P</i> value	CI (μM)
Control cells without insulin	12	227 ± 14		
Control cells with insulin	12	301 ± 25	<i>P</i> < 0.001 vs control cells without insulin	52–94
Control cells (Na ⁺ medium) with insulin and wortmannin (0.1 μmol/l)	6	165 ± 15	<i>P</i> < 0.01 vs control cells without insulin	17–59
1,2-Dioctanoyl-sn-glycerol (100 μmol/l) without insulin	4	305 ± 38	<i>P</i> < 0.01 vs control cells without insulin	40–87
1,2-Dioctanoyl-sn-glycerol (100 μmol/l) with insulin	4	335 ± 36	<i>P</i> < 0.001 vs control cells without insulin	50–97
Control cells (Na ⁺ medium) with vanadate (50 μmol/l)	3	78 ± 18		
Control cells (Na ⁺ medium) with vanadate (1 mmol/l)	6	135 ± 20	<i>P</i> < 0.003	50–120
Control cells (Na ⁺ medium) with insulin growth factor 1 (20 nmol/l)	6	230 ± 50		
Control cells (Na ⁺ medium) with 2-DOG (100 μmol/l)	6	570 ± 65	<i>P</i> < 0.0001	210–400
Control cells (Na ⁺ medium) with iodoacetic acid (300 μmol/l)	6	530 ± 60	<i>P</i> < 0.0001	100–270

All measurements were performed after 2.5 h; insulin when indicated is 10 μU/ml. Mean ± s.d. values of 3–12 independent experiments were provided; CI: 95% confidence limits. 2-DOG: 2-deoxy-D-glucose.

Table 2 Combined effect of insulin and inhibitors or regulators of transmembrane Mg^{2+} fluxes in human lymphocytes

Treatment	<i>n</i>	Intralymphocyte Mg_i^{2+} concentration (μM)	<i>P</i> value	CI (μM)
Control cells (Na ⁺ medium) without insulin	12	227 ± 14		
Control cells (Na ⁺ medium) with insulin	12	301 ± 25	<i>P</i> < 0.001 vs control cells without insulin	52–94
Choline Na ⁺ substitution medium without insulin	6	789 ± 51	<i>P</i> < 0.001 vs control cells without insulin	452–592
Choline Na ⁺ substitution medium with insulin	6	749 ± 12	<i>P</i> < 0.001 vs control cells with insulin	330–469
Insulin + 100 μmol/l bumetanide	9	300 ± 12	<i>P</i> < 0.001 vs control cells without insulin	49–100
500 μmol/l imipramine with insulin	6	230 ± 27		
500 μmol/l quinidine with insulin	6	240 ± 26		
Insulin + 0.1 mmol/l ouabain	9	297 ± 10	<i>P</i> < 0.001 vs control cells without insulin	48–95
0 mmol/l extracellular Mg^{2+} without insulin	4	206 ± 23		
0.8 mmol/l extracellular Mg^{2+} without insulin	4	238 ± 18		
3 mmol/l extracellular Mg^{2+} without insulin	4	231 ± 25		
0 mmol/l extracellular Mg^{2+} with insulin	4	243 ± 19	<i>P</i> < 0.05 vs 0 mmol/l extracellular Mg^{2+} without insulin	25–50
0.8 mmol/l extracellular Mg^{2+} with insulin	4	276 ± 20	<i>P</i> < 0.05 vs 0.8 mmol/l extracellular Mg^{2+} without insulin	20–60
3 mmol/l extracellular Mg^{2+} with insulin	4	271 ± 22	<i>P</i> < 0.05 vs 3 mmol/l extracellular Mg^{2+} without insulin	23–49

All measurements were performed after 2.5 h; insulin when indicated is 10 μU/ml; in experiments conducted with different extracellular magnesium concentrations a different medium was used (Bulher solution) as compared with the others (Na⁺ medium)—for details see Delva *et al.* (1998b). Mean ± s.d. values of 3–12 independent experiments were provided; CI: 95% confidence limits.

$147 \pm 22 \mu\text{M}$, ANOVA: $F=7.59$, $P=0.002$, respectively ($n=5$). In the presence and absence of $10 \mu\text{U/ml}$ of insulin, the Mg_i^{2+} concentrations relating to lymphocyte whole-cell Mg_i^{2+} , compartmentalized Mg_i^{2+} and cytosolic Mg_i^{2+} , were all statistically significantly different ($P=0.01$).

Under our experimental conditions, the incubation of lymphocytes for 2.5 h with $10 \mu\text{U/ml}$ of insulin did not produce any statistically significant modifications in the concentration of ATP_i , (control medium, $5.31 \pm 0.73 \text{ mM}$; insulin-treated, $5.78 \pm 0.85 \text{ mM}$, $n=6$).

Total intracellular magnesium in lymphocytes incubated in the medium without insulin was $19 \pm 2 \text{ mM}$ ($n=12$). Incubation of lymphocytes for 2.5 h with $10 \mu\text{U/ml}$ of insulin did not significantly modify this value (glucose-treated, $18.7 \pm 1.5 \text{ mM}$, $n=12$).

As far as ionized intracellular calcium is concerned, incubation of lymphocytes for 2.5 h with $100 \mu\text{U/ml}$ of insulin did not modify the concentration of Ca_i^{2+} in lymphocytes (without insulin, $60 \pm 11 \text{ nM}$, with insulin 65 ± 12 , $n=8$).

Discussion

Incubation of human lymphocytes with insulin at concentrations that mimic both physiological and pathological situations results in a significant increase in the Mg_i^{2+} concentration, confirming data obtained from anucleated human-derived cells such as erythrocytes (Barbagallo *et al.* 1993, 1997) and platelets (Hwang *et al.* 1993). However, in these previous studies, the interrelationships between intracellular magnesium, glucose and insulin were not investigated.

Paolisso *et al.* (1986) were the first to show in human erythrocytes that insulin *in vitro* increases erythrocyte magnesium, an effect that is entirely abolished by ouabain. The same authors then showed that the insulin-induced magnesium increase in erythrocytes is different in patients with type 2 diabetes (Paolisso *et al.* 1988) or obese subjects (Paolisso *et al.* 1990) compared with healthy subjects.

Dominguez *et al.* (1998) confirmed in human erythrocytes that insulin is capable of increasing intracellular free magnesium in a dose- and time-dependent manner (Barbagallo *et al.* 1993) and that IGF-1 has a similar effect.

Hwang *et al.* (1993) confirmed these results in a different cell model, namely the human platelet, and showed that insulin had a dose- and time-dependent effect on the increase of $[\text{Mg}^{2+}]_i$ and suggested that insulin can translocate Mg^{2+} from the extracellular space. Takaya *et al.* (1998) confirmed and extended the results on human platelets showing that IGF-1 produces the same increases in Mg_i^{2+} as insulin. The involvement of $\text{Na}^+-\text{Mg}^{2+}$ exchanger was judged as improbable and insulin/IGF-1 translocates Mg^{2+} from the extracellular space.

To our knowledge, only Hua *et al.* (1995) have studied the *in vitro* effects of insulin on human resting lymphocytes and confirmed the results obtained from erythrocytes and platelets

by demonstrating that insulin *in vitro* can increase Mg_i^{2+} in human resting lymphocytes. The results obtained from the present work are in agreement with the above results obtained from a different cell model utilizing different methodological approaches. As far as we know, the only study performed in insulin-target cells was performed in rat hepatocytes by Keenan *et al.* (1996), who demonstrated that insulin is able to block the Mg^{2+} efflux from perfused rat livers stimulated by isoproterenol.

We have previously studied the *in vitro* effect of glucose alone on Mg_i^{2+} in human lymphocytes and found that in the absence of insulin, glucose is capable of decreasing the Mg_i^{2+} in a dose-dependent and stereospecific manner (Delva *et al.* 2002). The glucose-induced increase in intracellular ATP, binding a greater amount of Mg_i^{2+} and forming MgATP, seems to be the most likely cause for the glucose-induced decrease in ionized magnesium (Delva *et al.* 2002).

In the present paper, we studied the *in vitro* effects of insulin, both alone or in combination with glucose, on lymphocyte Mg_i^{2+} . The latter situation, i.e. the interaction between insulin, glucose, and Mg_i^{2+} , which obviously tries to mimic the physiological situation, is complex. As shown in Fig. 1A, insulin is capable of significantly increasing the Mg_i^{2+} in all glucose concentrations tested, which were intended to mimic conditions of overt hyperglycemia (15 mmol/l), hyperglycemia (10 mmol/l) and fasting (5 mmol/l). From a kinetic point of view, the three dose-response curves are sigmoidal with approximately the same EC_{50} . The time courses of the insulin-induced increase in Mg_i^{2+} (Fig. 1B) and glucose-induced decreases in Mg_i^{2+} (Delva *et al.* 2002) are superimposable as far as the timing of initial and maximal effect are concerned. The effect of both insulin and glucose on intralymphocyte-ionized magnesium does not begin immediately, but takes place after about 30 min.

Regarding the mechanisms involved in the insulin-mediated increase of Mg_i^{2+} , the lack of effects of inhibitors of the membrane Na^+-K^+ pump (ouabain) and membrane $\text{Na}^+-\text{K}^+-\text{Cl}^-$ cotransport (bumetanide) on the insulin-mediated increase of Mg_i^{2+} is at odds with the possibility that these membrane transport systems are involved. The substitution of extracellular sodium with choline, which blocks the membrane $\text{Na}^+-\text{Mg}^{2+}$ exchanger, abolishes the insulin-mediated increase of Mg_i^{2+} . However, since the method for blocking the $\text{Na}^+-\text{Mg}^{2+}$ exchanger increases cytosolic Mg^{2+} to a very high level and may even be toxic, we used two well-known inhibitors of the $\text{Na}^+-\text{Mg}^{2+}$ exchanger: imipramine and quinidine (Feraý and Garay 1986). In the presence of either inhibitor, no increase in Mg_i^{2+} was induced by insulin. This is in favor of the hypothesis that insulin is capable of increasing the Mg_i^{2+} by inhibiting this membrane transport.

In contrast with previous data (Romani *et al.* 2000), insulin is capable of increasing the Mg_i^{2+} even in the absence of extracellular glucose or magnesium. This latter fact would exclude that an $\text{Ca}_o^{2+}-\text{Mg}_i^{2+}$ exchanger or an inwardly directed Mg^{2+} channel is involved in the process. By

increasing the extracellular magnesium from 0 to 3 mmol/l, the Mg_i^{2+} increases progressively both in the absence and presence of insulin. In fact, the two curves are parallel, which, together with the lack of modification of total intracellular magnesium in the presence of insulin, suggests that the insulin-induced increase in Mg_i^{2+} is not due to increases in passive permeability. Data obtained with differential membrane permeabilization do not seem to localize the insulin-mediated increase of Mg_i^{2+} either in the cytosol or in cellular organelles.

In order to better understand the mechanism of the insulin effects on Mg_i^{2+} , we evaluated molecules characterized by an insulin-mimicking action such as IGF-1 and vanadate. Dominguez *et al.* (1998) were the first to test *in vitro* the effects of IGF-1 on intracellular magnesium. IGF-1 is capable of increasing Mg_i^{2+} in erythrocytes from normotensive and hypertensive subjects. The results in which no effects of IGF-1 could be detected on lymphocyte Mg_i^{2+} are difficult to compare to those obtained by Dominguez *et al.* (1998) because different cell models and analytical methods were used. Barbagallo *et al.* (2001) have been the only investigators to assess the effect of vanadate on intracellular magnesium compared to the effects of insulin. In the experimental conditions utilized (in the erythrocyte measuring with nuclear magnetic resonance), vanadate increased Mg_i^{2+} in a manner that was comparable to insulin at a concentration of 50 μ mol/l, while at a concentration of 1 mmol/l, it had the opposite effect and produced a decrease. We tested two concentrations of vanadate on lymphocytes. At high concentrations (1 mmol/l), it decreased Mg_i^{2+} , while at 50 μ mol/l, vanadate did not show any effect on lymphocyte magnesium. The discrepancy of the effects of vanadate on Mg_i^{2+} in lymphocytes with respect to erythrocytes is difficult to reconcile with the current cellular model and the different methods of measurement. However, if this were the case, our results would also be different for insulin, which is in agreement with published studies. Our data suggests that in lymphocytes, vanadate does not mimic insulin action. The decrease in Mg_i^{2+} seen at high concentrations may be attributable to the toxicity of vanadate on various membrane transport systems (Domingo 2002).

PI 3-kinase is known to be a part of the signaling pathways for Na^+ transporters. In adipose tissue, PI 3-kinase has been shown to be activated by insulin and to mediate the effect of insulin on glucose transport. To examine the role of PI 3-kinase on insulin-induced Mg_i^{2+} increase, we measured ionized magnesium in lymphocytes incubated with insulin in the presence and absence of wortmannin (WT), an established inhibitor of PI 3-kinase. Incubation of lymphocytes with 100 μ U/ml insulin and 0.1 μ mol/l WT completely blocked the insulin-stimulated Mg_i^{2+} increase, suggesting yet another role for the PI 3-kinase pathway in insulin-mediated cellular events. Ferreira *et al.* (2003) showed that insulin-stimulated Mg^{2+} efflux from Mg^{2+} -loaded erythrocytes is blocked by WT. Despite this, an insulin-stimulated Mg_i^{2+}

magnesium efflux does not explain the insulin-stimulated Mg_i^{2+} increase that we observed.

The use of 1,2-dioctanoyl-sn-glycerol, a cell-permeable diacylglycerol analog, mimics the effect of insulin on Mg_i^{2+} , suggesting that insulin may act on intracellular Mg_i^{2+} at least in part by activation of protein kinase C (PKC).

Finally, we utilized iodoacetic acid to inhibit the hexose monophosphate shunt, and 2-deoxy-D-glucose to inhibit glycolysis. Both substances greatly increased Mg_i^{2+} , as shown in Table 1, and we propose that it is likely to be due to a decrease in intracellular ATP and the consequent increase in free, unbound magnesium. The massive increase of ionized intracellular magnesium produced by both these substances prevents the measurement of any additional effects of insulin.

Even though the calcium determination was not the main aim of the study, intralymphocyte-free calcium was also measured, mainly to exclude substantial intracellular calcium increase that may theoretically affect magnesium determinations (Hurley *et al.* 1992). Insulin did not alter the Ca_i^{2+} , thus confirming the results obtained *in vitro* in other cell models (Thomas *et al.* 1985).

In conclusion, we have shown for the first time in human nucleated cells the ability of insulin to modify *in vitro* the concentration of Mg_i^{2+} through a mechanism based on activation of a Na^+ - Mg^{2+} membrane transport system, possibly through the mediation of PI 3-kinase and PKC activation. Taking into account the relationship between insulin and glucose plasma levels and their opposite effects on Mg_i^{2+} , this may represent the two limbs of a biphasic regulatory system of Mg_i^{2+} in both physiological and pathological conditions. An increase of insulin in response to eating, and therefore before an increase in plasma glucose levels, can be hypothesized, increasing Mg_i^{2+} and augmenting the activity of enzymes involved in glucose metabolism to bring about conditions that are more appropriate for metabolism and production of energy. Moreover, this effect would be produced as a final result of the increased enzymatic velocity and decrease in the extracellular concentration of glucose to reestablish glucose homeostasis. The decrease in extracellular glucose thus obtained would bring about a decrease in insulinemia that would precede a decrease in Mg_i^{2+} , with reestablishment of cellular magnesium. In type 1 or 2 diabetes mellitus, the elevated plasma glucose concentrations in the absence of insulin or in situations of peripheral resistance to its actions would produce a prevailing effect, decreasing the inhibition of Mg_i^{2+} . This would lead to a slowing of glucose degradation and would negatively influence magnesium-dependent enzymatic reactions. In addition, it would explain the frequent observation of reduced intracellular magnesium concentrations in patients with diabetes mellitus (Paolisso *et al.* 1988).

It is clear that this mechanism, which has been shown in lymphocytes, cannot easily be extrapolated to other

cellular models that are much more important in the physiopathology of glucose metabolism, both in physiological conditions and in diabetes mellitus. Despite this, a direct correlation has been described between the concentration of magnesium in lymphocytes and that in striated muscular tissue (Dyckner and Wester 1985). It must also be remembered that some authors have shown that there is an alteration of pyruvate dehydrogenase (Curto *et al.* 1997) in the lymphocytes of patients with type 2 diabetes mellitus, which is in agreement with studies carried out in adipocytes (Mandarino *et al.* 1986) and in skeletal muscle (Kelley *et al.* 1992) of patients with type 2 diabetes mellitus. Finally, several investigators have described the presence of GLUT3 in human lymphocytes (Estrada *et al.* 1994), which is a transporter in some cell types that have been shown to be insulin sensitive (Bilan *et al.* 1992). Estrada *et al.* (1994) have also demonstrated that in contrast to monocytes, lymphocytes show a significant increase in the expression of GLUT3 in patients with insulin-dependent diabetes mellitus with respect to healthy control subjects.

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