Role of Extracellular Magnesium in Insulin Secretion from Rat Insulinoma Cells (43459)

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Abstract. **Magnesium (Mg^{2+}) is an abundant intracellular cation that participates in the regulation of the intracellular concentration of ATP. In this study, we examined the relationship between insulin secretion and intracellular free Mg^{2+} ($[Mg^{2+}]^i$) in a rat-insulinoma cell line (RIN m5F), using a fluorescent dye (Mag-fura-2). KCl, forskolin, and D-glyceraldehyde increased $[Mg^{2+}]^i$ and insulin secretion from RIN m5F cells in a dose-dependent fashion. Verapamil, a voltage-dependent Ca^{2+} channel blocker, inhibited the increase of $[Mg^{2+}]^i$ that was evoked by KCl, forskolin, and D-glyceraldehyde. In a Mg^{2+}-free buffer, these agents failed to cause an elevation in $[Mg^{2+}]^i$; however, the insulin response to KCl and forskolin was enhanced, compared with that in the presence of Mg^{2+} (1.25 m*M*). Our findings suggest that $[Mg^{2+}]^i$ is dependent upon extracellular Mg^{2+}, and the influx through the voltage-dependent Ca^{2+} channel. Mg^{2+} may competitively inhibit the voltage-dependent Ca^{2+} channel, which is known to play a role in insulin secretion. An absence of Mg^{2+} in the extracellular space may result in enhanced insulin secretion. $[Mg^{2+}]^i$ may play a role in insulin secretion from RIN m5F cells.**

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Magnesium (Mg^{2+}), the most abundant intracellular divalent cation, is known to be an essential cofactor for many enzymes and to play an essential role in protein synthesis in a variety of cells (1-4). Furthermore, Mg^{2+} can stabilize ribosomal and membrane structures and is considered essential for protein insertion into membranes (1-4). Exactly how Mg^{2+} moves into a cell (which may be crucial in order to keep intracellular ($[Mg^{2+}]^i$) levels constant) remains controversial (5-7).

The purpose of this study was to examine the Mg^{2+}-influx pathway in an insulinoma cell line (RIN m5F) and to evaluate the role of magnesium in insulin secretion from RIN m5F cells. We used Mag-fura-2/acetoxymethyl ester (Mag-fura-2AM), an intracellular magnesium indicator, to measure $[Mg^{2+}]^i$ (8).

Materials and Methods

Mag-fura-2AM and fura-2AM were purchased from Molecular Probes, Inc., (Eugene, OR). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

RIN m5F cells were maintained in 75-cm^2 plastic culture flasks with RPMI 1640 medium containing glucose (11.1 m*M*) and 10% fetal calf serum without antibiotics at 37°C in humidified 5% CO_2 and 95% air. Cells were dispersed using 0.1% trypsin in RPMI 1640 and were plated at a density of 10^6 cells/flask every week (9).

$[Mg^{2+}]^i$ was measured using a magnesium indicator (Mag-fura-2AM). For Mag-fura-2 experiments, 1×10^7 cells were resuspended from stock cultures and incubated for 60 min at 18°C in 30 ml of Krebs-Ringer bicarbonate buffer (KRBB; pH 7.4) containing Mg^{2+} (1.25 m*M*) and Mag-fura-2AM (2 m*M*). Loaded cells were washed twice with 1 ml of KRBB and maintained at 18°C until the experiment. Approximately 1×10^5 cells/ml were suspended in 3.0 ml of KRBB in a cuvette. Fluorescence was recorded with a dual-wavelength fluorometer (Spex Industries, Inc., Edison, NJ)

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at excitation wavelengths of 340 nm (F340) and 380 nm (F380) and an emission wavelength of 505 nm. In these experiments, intracellular magnesium was calibrated by lysing the cells with 0.03% Triton X-100 and estimating the maximal and minimal Mag-fura-2 fluorescence using 1.5 m*M* as the K_d for magnesium. The intracellular magnesium concentration was calculated by the method of Grynkiewicz and colleagues (10). KRBB containing 5 m*M* magnesium and magnesium-free KRBB were made by adding and removing $MgCl_2$ from the KRBB, respectively.

Intracellular calcium concentrations ($[Ca^{2+}]^i$) were measured using fura-2AM, a calcium indicator. The method was similar to that using Mag-fura-2AM, except that the intracellular calcium concentration was calculated using 224 n*M* as the K_d for calcium (10).

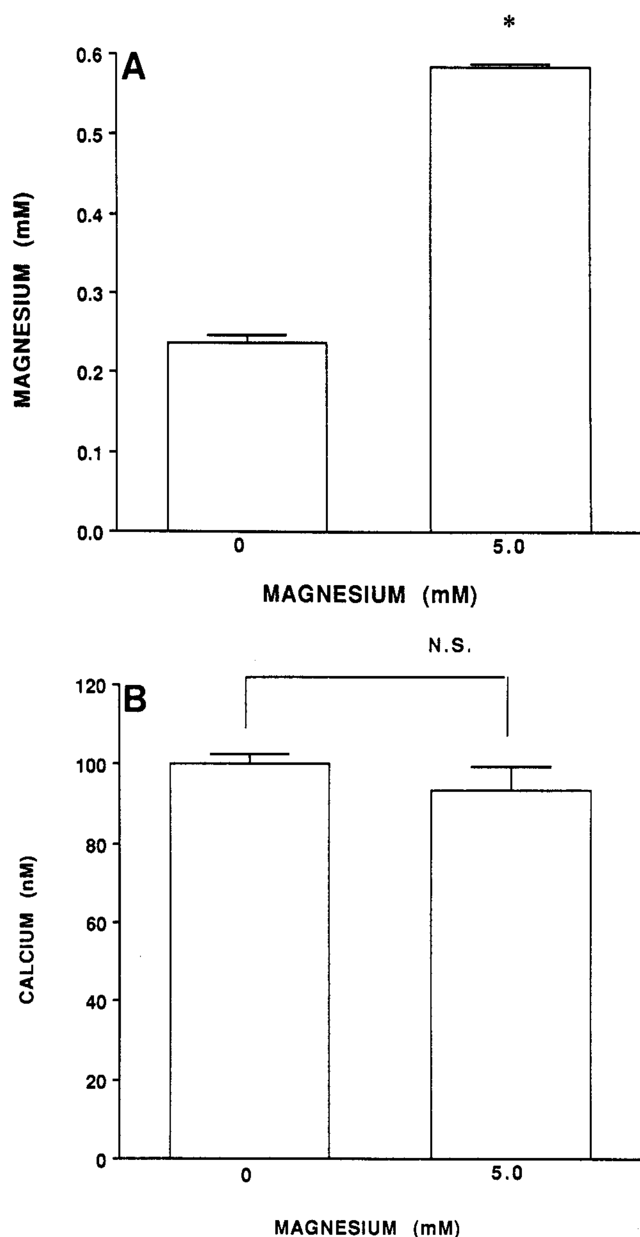

Figure 1. Intracellular magnesium or calcium changes induced by extracellular magnesium. (A) Changes in intracellular magnesium induced by 5 m*M* magnesium buffer. (B) Changes in intracellular calcium induced by 5 m*M* magnesium. *$P < 0.05$ vs 0 m*M* Mg^{2+}; NS, not significant.

For insulin secretion experiments, 2 ml of 1×10^5 cells/ml were seeded to each well of a 24-well plate and cultured for 2 days. At the time of the experiment, cells were preincubated for 30 min at 37°C with 1 ml of KRBB containing 2.8 m*M* glucose and 0.1% bovine serum albumin. Cells were then washed twice with 1 ml of KRBB and incubated with 1 ml of KRBB containing 1.25 m*M* Mg^{2+} and stimulants for 30 min at 37°C. KCl, forskolin, and D-glyceraldehyde were used as secretagogues. The concentration of sodium in the buffer was lowered to compensate for KCl. The effect of glibenclamide, an ATP-dependent K^+-channel blocker, on insulin secretion in the Mg^{2+}-free condition was also examined to analyze whether the effect of magnesium on the ATP-dependent K^+-channel was related to insulin secretion data. Supernatants were collected and stored at −20°C until radioimmunoassay of insulin (11).

Statistical differences were identified and estimated by Duncan's *t* test (12). Data are expressed as mean ± SE. A *P*-value less than 0.05 was considered significant.

Results

Changes of $[Mg^{2+}]^i$ and $[Ca^{2+}]$ in RIN m5F cells. A total of 1.25 m*M* Mg^{2+} did not affect $[Mg^{2+}]^i$. A total of 5 m*M* of Mg^{2+} increased $[Mg^{2+}]^i$, but did not affect $[Ca^{2+}]^i$ (Fig. 1, A and B). KCl, forskolin, and D-glyceraldehyde increased $[Mg^{2+}]^i$ in a dose-dependent fashion, whereas in Mg^{2+}-free KRBB, neither KCl, forskolin, nor D-glyceraldehyde increased $[Mg^{2+}]^i$ (Table I). Time course changes in response to KCl (90 m*M*) are shown

Table I. Intracellular Magnesium Concentration (μ*M*)

	Normal KRBB	Mg^2-free KRBB	Verapamil (25 m*M*)
KCl			
5 m*M*	234 ± 10	243 ± 6	202 ± 11
30 m*M*	384 ± 10[a]	245 ± 6	197 ± 12
45 m*M*	456 ± 10[a]	251 ± 9	201 ± 7
90 m*M*	535 ± 9[a]	250 ± 9	198 ± 6
D-Glyceraldehyde			
0 m*M*	244 ± 9	238 ± 6	209 ± 12
0.1 m*M*	318 ± 8[b]	232 ± 9	199 ± 12
1.0 m*M*	363 ± 6[b]	232 ± 9	190 ± 7
10 m*M*	419 ± 10[b]	249 ± 12	215 ± 15
Forskolin			
0 m*M*	243 ± 10	236 ± 4	195 ± 7
0.01 m*M*	292 ± 9[c]	233 ± 3	178 ± 7
0.1 m*M*	331 ± 8[c]	237 ± 9	182 ± 5
1 m*M*	375 ± 10[c]	236 ± 12	187 ± 4

[a] $P < 0.05$ vs 5 m*M*.
[b] $P < 0.05$ vs 0 m*M*.
[c] $P < 0.05$ vs 0 m*M*.

in Figure 2A. KCl (90 mM) increased F340 and decreased F380 simultaneously. Changes in the intracellular concentration of magnesium, which were calculated at F340/F380 in response to KCl (90 mM), are shown in Figure 2B. KCl (90 mM) increased $[Mg^{2+}]^i$ quickly. After the peak level, $[Mg^{2+}]^i$ decreased slightly and sustained. Verapamil, a voltage-operated Ca^{2+} channel (VOC) antagonist, inhibited increases of $[Mg^{2+}]^i$ in response to KCl, forskolin, and D-glyceraldehyde (Table I).

Insulin Secretion. In Mg^{2+}-free KRBB, D-glyceraldehyde did not affect insulin secretion compared with that in a condition of 1.25 mM Mg^{2+} (Fig. 3A). Forskolin (10^{-6} M) significantly increased insulin secretion compared with that in normal KRBB (1.25 mM Mg^{2+}) (Fig. 3B). Insulin secretion in response to 30 or 45 mM KCl was significantly higher in Mg^{2+}-free KRBB, but 90 mM KCl-induced insulin secretion showed no significant difference (Fig. 3C). In glibenclamide (10 mM)-containing buffer, insulin secretion in response to 30 mM KCl was higher in Mg^{2+}-free KRBB (Fig. 4).

Discussion

In this study, we used Mag-fura-2, an indicator insensitive to intracellular pH and to the presence of Ca^{2+} in the physiologic range; Mag-fura-2 also exhibits fluorescence changes depending upon concentration with magnesium in a similar fashion to those observed in concentrations of Ca^{2+} with fura-2 (8). The acetoxymethyl form of this indicator is readily loaded into cells (7). We measured intracellular free magnesium concentration with this new intracellular magnesium

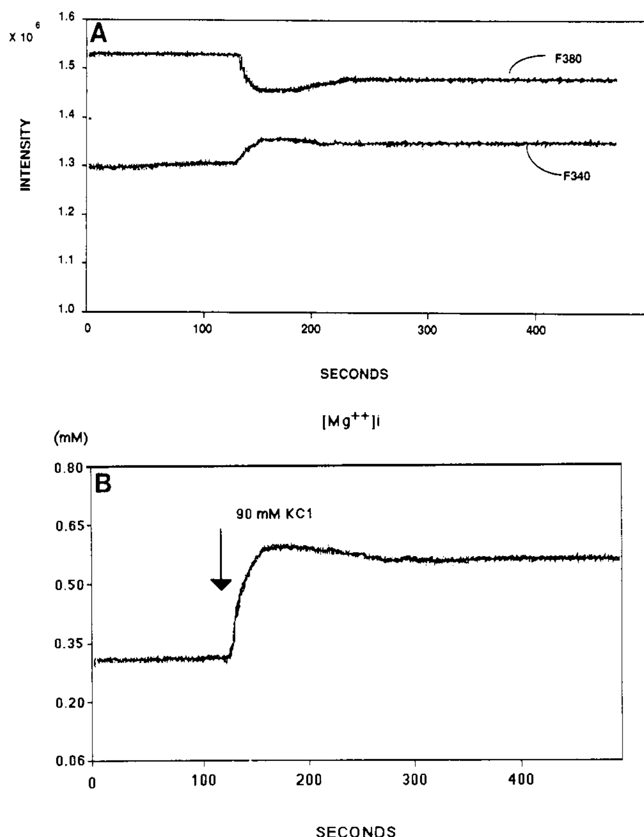

Figure 2. Changes in intracellular magnesium. Time-course change induced by 90 mM KCl. (A) Fluorescence intensity of the F340 and F380. (B) Ratio of F340 to F380 is calculated and the magnesium concentration is shown. Data are representative of six experiments.

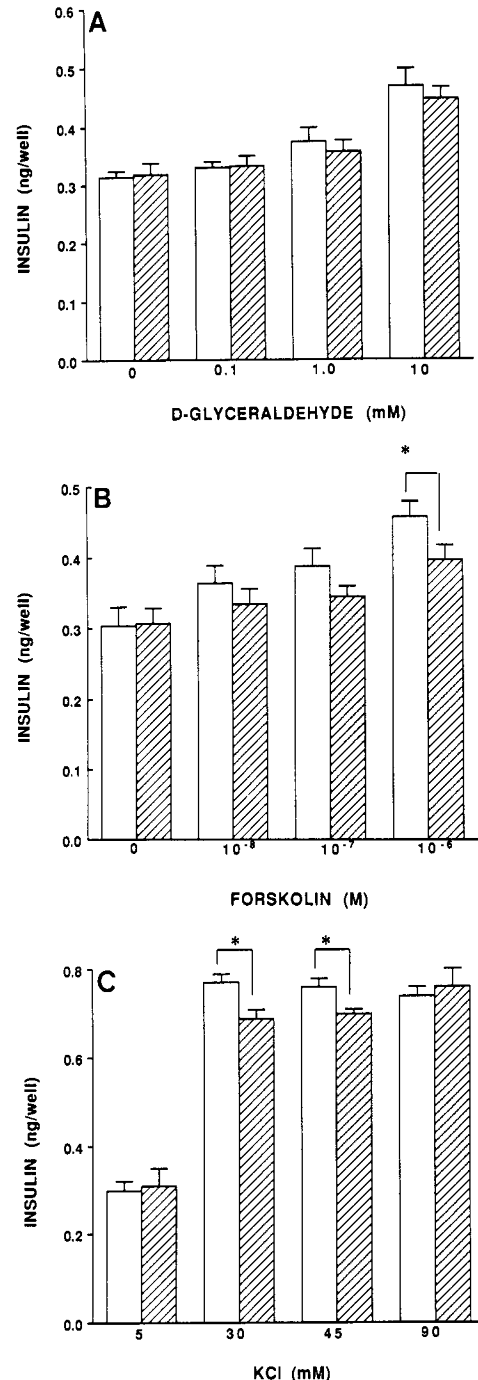

Figure 3. Insulin secretion induced by (A) D-glyceraldehyde, (B) forskolin, and (C) KCl with or without extracellular magnesium (1.25 mM). Open bars show responses of insulin secretion in magnesium-free buffer; hatched bars show responses of insulin secretion in normal buffer (1.25 mM magnesium). *$P < 0.05$ vs normal buffer.

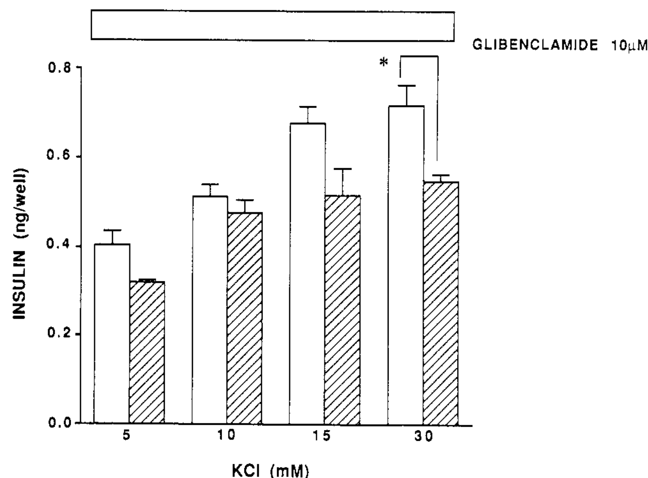

Figure 4. Insulin secretion induced by KCl with or without extracellular magnesium (1.25 m*M*) in glibenclamide (10 m*M*)-containing buffer. Open bars show the effect of KCL on insulin secretion in magnesium-free buffer; hatched bars show the effect of KCl on insulin secretion in normal buffer (1.25 m*M* magnesium) $^{*}P < 0.05$ vs normal buffer.

indicator and found that it was independent of intracellular calcium changes.

KCl and D-glyceraldehyde are known to depolarize the membranes of RIN m5F cells. Also, cAMP is thought to increase Ca^{2+} influx through the VOC. KCl, D-glyceraldehyde, and forskolin increased $[Mg^{2+}]^i$. Verapamil, a blocker of the VOC, inhibited intracellular magnesium changes in response to KCl, D-glyceraldehyde, and forskolin. In the magnesium-free buffer, $[Mg^{2+}]^i$ did not change in response to these stimulants. Present findings suggest that the Mg^{2+} influx pathway is voltage dependent and verapamil sensitive. Quamme and Rabkin (7) reported that extracellular magnesium did not affect $[Ca^{2+}]^i$ in cardiac myocytes using the same indicator, but our data suggest that the system of magnesium influx shares the same pathway with calcium influx in RIN m5F cells. To examine the effect of magnesium on insulin secretion, we examined insulin secretion with Mg^{2+}-free KRBB or Mg^{2+} containing KRBB.

The fact that insulin secretion increased in response to KCl and forskolin in Mg^{2+}-free KRBB suggests two possible mechanisms. First, because Mg^{2+} is thought to be a negative regulator of intracellular free-ATP and ADP concentrations, and since free ATP is known to regulate ATP-dependent K^+ channels (13), the increased intracellular free ATP concentration induced by Mg^{2+}-free KRBB closes the ATP-dependent K^+ channel, which results in a rise in the membrane potential that increases insulin secretion.

To further understand the contribution of Mg^{2+}-related ATP-dependent K^+ channels on insulin secretion, we used glibenclamide, a blocker of ATP-dependent K^+ channels. We found it possible, in this experiment, to examine the effect of Mg^{2+} on the VOC separately. Our results suggest that the effect of magnesium on insulin secretion is not mediated through its antagonistic action on the ATP-dependent K^+ channels.

The second putative mechanism is that since the Mg^{2+} influx shares a common pathway with the Ca^{2+} influx, Mg^{2+} may act as a competitive antagonist for the Ca^{2+} influx mediated through the VOC. Baker (14, 15) reported that Mg^{2+} could influence hormone release from adrenal medullary cells not only by competing with calcium influx, but also at intracellular sites controlling exocytosis, indicating that there may be other Mg^{2+} mechanisms that affect the mechanism of insulin secretory.

The concentration of $[Mg^{2+}]^i$ is maintained below the electrochemical equilibrium, which indicates that there are active Mg^{2+} transport systems (16). To study the active Mg^{2+} transport system and the Mg^{2+} efflux system, we employed: KCl, which increases the membrane potential; forskolin, which is an activator of adenylate cyclase; and D-glyceraldehyde, which is known as a metabolite of glucose and induces insulin secretion (17). Our findings suggest that the Mg^{2+} efflux system is not voltage dependent, and that cAMP and D-glyceraldehyde have no effect on the Mg^{2+} efflux system because $[Mg^{2+}]^i$ did not change to these stimulants in Mg^{2+}-free KRBB. The active Mg^{2+} transport system and the Mg^{2+} efflux system may be regulated by another mechanism in RIN m5F cells.

We conclude that Mg^{2+} regulates insulin secretion through the voltage-dependent mechanism in RIN m5F cells. Extracellular Mg^{2+} may play a role in insulin secretion by acting on the VOC. The effect of magnesium on the regulation of ATP concentration is thought to be less important than its effect on the VOC.

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