Streptozotocin-induced diabetes impairs Mg²⁺ homeostasis and uptake in rat liver cells

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Fagan, Theresa E., Christie Cefaratti, and Andrea Romani. Streptozotocin-induced diabetes impairs Mg²⁺ homeostasis and uptake in rat liver cells. Am J Physiol Endocrinol Metab 286: E184–E193, 2004; 10.1152/ajpendo.00200.2003.—Male Sprague-Dawley rats rendered diabetic by streptozotocin injection presented 10 and 20% decreases in total hepatic Mg²⁺ content at 4 and 8 wk, respectively, following diabetes onset. This decrease was associated with a parallel decrease in K⁺ and ATP content and an increase in Na⁺ level. In diabetic liver cells, the Mg²⁺ extrusion elicited by α₁-adrenoceptor stimulation was markedly reduced compared with nondiabetic livers, whereas that induced by β-adrenoceptor stimulation was unaffected. In addition, diabetic hepatocytes did not accumulate Mg²⁺ following stimulation of protein kinase C pathway by vasopressin, diacylglycerol analogs, or phorbol 12-myristate 13-acetate derivate despite the reduced basal content in cellular Mg²⁺. Experiments performed in purified plasma membrane from diabetic livers located the defect at the level of the bidirectional Na⁺/Mg²⁺ exchanger operating in the basolateral domain of the hepatocyte cell membrane, which could extrude but not accumulate Mg²⁺ in exchange for Na⁺. The impairment of Mg²⁺ uptake mechanism, in addition to the decrease in cellular ATP level, can contribute to explaining the decrease in liver Mg²⁺ content observed under diabetic conditions.

Magnesium; adrenergic signaling; protein kinase c; adenosine 5'-triphosphate; plasma membrane

MAMMALIAN CELLS regulate Mg²⁺ homeostasis and transport across the cell membrane via complex mechanisms that are under tight hormonal control (14, 32). Cardiac myocytes (39, 31), hepatocytes (9, 27), erythrocytes (16, 20), and lymphocytes (41) all extrude a sizable amount of cellular Mg²⁺ as a result of the increase in cAMP that follows the activation of β-adrenergic (27, 31, 39), glucagon (9), or progesterone receptors (41) or the administration of cell-permeant cAMP analogs (i.e., 8-Cl-cAMP or dibutyryl-cAMP) (27, 31, 39) or forskolin (27, 31), an agent that activates adenyl cyclase. Further evidence for a role of cAMP in eliciting Mg²⁺ extrusion is provided by the effect of the Rp-cAMP isomer, which stably blocks adenylyl cyclase and prevents cAMP-mediated Mg²⁺ extrusion (41). Liver cells can also extrude Mg²⁺ following activation of α₁-adrenoceptor (9, 10). Under all these conditions, Mg²⁺ is transported across the cell membrane via a putative Na⁺/Mg²⁺ exchanger (reviewed in Ref. 13). Although the transporter has not been cloned to date, the Na⁺ dependence of Mg²⁺ extrusion is supported by results in various experimental models, which indicate that Mg²⁺ extrusion is largely inhibited in the absence of extracellular Na⁺ (29, 30) or in the presence of the Na⁺ transport inhibitors amiloride (15) and imipramine (12).

Mammalian cells can also accumulate Mg²⁺ as a result of protein kinase C activation or a decrease in cAMP level. Agents such as carbachol or insulin that decrease cellular cAMP level, in fact, induce a marked accumulation of Mg²⁺ within liver cells (28), cardiac myocytes (28, 31), platelets (36), or fibroblasts (17). Activation of protein kinase C by cell-permeant analogs of diacylglycerol (25), derivate of phorbol 12-myristate 13-acetate (PMA) (28), or hormones like vasopressin (27) also results in an increase in cellular Mg²⁺ content. It is presently undefined whether Mg²⁺ entry is mediated by the reverse operation of the Na⁺/Mg²⁺ exchanger, as suggested by data obtained in purified liver plasma membranes (5, 6), or a distinct pathway, possibly a channel, as indicated by results in cardiac myocytes (25) or kidney cells (24).

Because of the relative recent interest in Mg²⁺ homeostasis, scarce data are available about defects in Mg²⁺ transport and regulation under pathological conditions. Experimental and clinical evidence, however, indicates that a loss of tissue and plasma Mg²⁺ is observed under both type 1 and type 2 diabetes (26, 40). The modulation by which this decrease in tissue and plasma Mg²⁺ occurs is largely unknown, as it is unclear to what extent the decrease in Mg²⁺ content is responsible for the onset of short- and long-term complications of diabetes.

The present study is the first to investigate the changes in hepatic Mg²⁺ homeostasis and transport under type 1 diabetic conditions. The results reported here indicate that the lack of insulin results in a time-dependent loss of Mg²⁺ from liver cells and possibly other tissues as well. In the hepatocyte, this loss is consequent to a decrease in cellular ATP content and the inability of the cell to accumulate Mg²⁺ following specific hormonal stimuli and restore cellular Mg²⁺ homeostasis.

MATERIALS AND METHODS

Induction of diabetes. Male Sprague-Dawley rats (200–220 g body wt) were rendered diabetic by a single intraperitoneal injection of 65 mg/kg body wt of streptozotocin in citrate buffer (pH 4.0). Urinary levels of glucose were measured by glucose strips (Fisher, Pittsburgh, PA) twice a week to monitor onset and progression of diabetes. The incidence of diabetes was determined as the appearance of glucose in the urine, which occurred within 36 h of streptozotocin administration. During the period of this study, no insulin supplement was provided. The animals were maintained on a 12:12-h light-dark cycle and had free access to food and water. Fewer than 5% of the animals injected with streptozotocin did not become diabetic. These animals were used as additional controls to exclude the possibility that the observed effects were due to streptozotocin metabolism.

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**Determination of tissue ion content.** At 4 and 8 wk after diabetes induction, diabetic and age-matched nondiabetic rats were anesthetized by intraperitoneal injection of a saturated pentobarbital sodium solution (50 mg/kg body wt). The abdomen was opened and the liver removed, rinsed in 250 mM sucrose, blotted on absorbing paper, weighed, and homogenized in 10% HNO₃. After overnight digestion, aliquots of the acid extracts were transferred into microfuge tubes, and the denatured protein was sedimented at 8,000 g for 5 min. The acid supernatants were removed and assessed for Mg²⁺, Ca²⁺, Na⁺, and K⁺ content by atomic absorption spectrophotometry (AAS) in a Perkin-Elmer S1000 after proper dilution. Cation content was normalized for protein content, measured according to the procedure of Lowry et al. (19) with bovine serum albumin as a standard.

**Liver perfusion.** Four- and eight-week diabetic and age-matched nondiabetic rats were anesthetized as indicated above. The abdomen was opened and the portal vein cannulated. The liver was perfused with a medium containing (in mM) 120 NaCl, 3 KCl, 1 CaCl₂, 0.8 MgCl₂, 1.2 K₂HPO₄, 12 NaHCO₃, 15 glucose, 10 HEPES, pH 7.2, at 37°C, equilibrated with an O₂-CO₂ (95:5 vol/vol) gas mixture. The liver was quickly removed from the abdominal cavity, placed on a platform, and perfused at a flow rate of 3.5–4 ml·g⁻¹·min⁻¹. After a few minutes of equilibration, the perfusion medium was switched to one having a similar composition but devoid of Mg²⁺ (Mg²⁺-free medium). The contaminant Mg²⁺ content of the perfusate was collected at 30-s intervals, and the Mg²⁺ content in the perfusate was measured by AAS. The first 10 min provided a baseline for the subsequent addition of adrenergic agonist. Phenylephrine (5 μM) or isoproterenol (10 μM) was dissolved directly into the perfusion medium and administered for the time reported in the figures. Pharmacological doses of the agonists were used to exclude reduced adrenoceptor responsiveness. To estimate the total amount of Mg²⁺ extruded from the organ, the Mg²⁺ content in the perfusate of the last six points before the adrenergic agonist addition was averaged and subtracted from each of the time points under the curve of efflux. The net amount of Mg²⁺ mobilized into the perfusate (nmol/ml) was then calculated, taking into account the perfusion rate (3.5–4 ml·g⁻¹·min⁻¹) and the time of collection (30 s), and expressed as micromoles. The residual Mg²⁺ content of perfused livers was also calculated in tissue homogenate, as described previously. The absence of cell damage was assessed by enzymatically measuring lactate dehydrogenase (LDH) activity in aliquots of the perfusate at 1-min intervals throughout the experimental procedure. The release of K⁺ from potentially damaged cells was also measured by AAS in aliquots of the perfusate (9, 31).

**Hepatocyte isolation.** Hepatocytes were isolated by collagenase digestion according to the procedure of Seglen (34). After isolation, the hepatocytes were resuspended in a medium having a composition similar to that reported in the previous section, containing 0.8 mM MgCl₂, and kept at room temperature, under constant flow of O₂-CO₂ (95:5) until used. Cell viability, assessed by Trypan blue exclusion test, was found to be 90 ± 3, n = 9, and did not change significantly over the course of 4 h (88 ± 4, n = 8).

To determine Mg²⁺ transport, 1 ml of cell suspension was transferred to a microfuge tube, and the cells were rapidly sedimented at 600 g for 30 s and washed with 1 ml of the Mg²⁺-free medium described above. After the washing, the cells were transferred to 8 ml of Mg²⁺-free incubation medium, prewarmed at 37°C, and incubated under continuous O₂-CO₂ flow and stirring. After 2 min of equilibration, agonists for α₁- or β-adrenergic receptor or for protein kinase C signaling were added to the incubation system. At the time reported in the figures, 0.7 ml of incubation mixture was withdrawn in duplicate, and the cells were sedimented in microfuge tubes. The supernatants were removed, and their Mg²⁺ content was determined by AAS. The cell pellets were digested overnight in 10% HNO₃, and the Mg²⁺ content of the acid extract was measured by AAS after sedimentation of the denatured protein in microfuge tube at 8,000 g for 5 min.

In a separate set of experiments, hepatocytes were incubated in the presence of 0.5 or 0.8 mM extracellular Mg²⁺. At selected times, aliquots of the incubation mixture were withdrawn in duplicate as reported above and sedimented in microfuge tubes through an oil layer containing dibutyl phthalate-diocyl phthalate (2:1 vol/vol) to remove excess extracellular Mg²⁺. The supernatant and oil layer were removed by vacuum suction, and the cell pellet was digested overnight in 0.5 ml of 10% HNO₃. The acid mixture was sonicated for 20 min, and the denatured protein was sedimented at 8,000 g for 5 min. The Mg²⁺ content of the acid supernatant was measured by AAS.

**Additional determinations.** Aliquots of the perfusate were collected every minute, and glucose content was determined by enzymatic kit (Sigma) as the variation in hexokinase-coupled NADH reduction. Additional determinations included the measurement of Mg²⁺ content by atomic absorbance spectrophotometry (AAS) in a Perkin-Elmer S1000 after proper dilution. Cation content was normalized for protein content, measured according to the procedure of Lowry et al. (19) with bovine serum albumin as a standard.

**Plasma membrane purification.** Total liver plasma membrane (tLPM) vesicles from diabetic and nondiabetic rats were isolated and stored as described in detail elsewhere (5). Plasma membrane purity was assessed by using 5'-nucleotidase, cytochrome c oxidase, and glucose-6-phosphatase activities as markers for plasma membrane, mitochondria, and endoplasmic reticulum, respectively (5). Negligible levels of cytochrome c oxidase and glucose-6-phosphatase activities were detected in both diabetic and nondiabetic tLPM compared with total liver homogenate. A fourfold enrichment in 5'-nucleotidase activity compared with total homogenate was observed in both preparations. Total tLPM orientation, determined by measuring Na⁺/K⁺-ATPase and 5'-nucleotidase activities (5), indicated that ≥90% of diabetic and nondiabetic tLPM loaded with Mg²⁺ were in the “inside-in” configuration. Na⁺/K⁺-ATPase activity in diabetic tLPM was ~20% of that measured in nondiabetic tLPM (0.034 ± 0.001 vs. 0.18 ± 0.02 μmol P·mg protein⁻¹·min⁻¹, respectively; n = 7, P < 0.05).

**Loading of LPM.** Aliquots of tLPM were loaded with 20 mM Mg²⁺ or Na⁺, according to our published protocols (1). The Mg²⁺-loaded vesicles were resuspended in 5 ml of 250 mM sucrose and 25 mM K-HEPES, pH 7.4, in the absence of added Mg²⁺ (Mg²⁺-free medium) and stored in ice until used. Loading efficiency was assessed by treating the vesicles with ionophore (A-23187) or detergent ( Triton X-100) and measuring the amount of Mg²⁺ extruded in the extravesicular space or retained within the vesicle pellet by AAS (1).

**Measurement of Mg²⁺ fluxes.** Mg²⁺-loaded tLPM were incubated in Mg²⁺-free medium as described above, at 37°C under continuous stirring, at the final concentration of ~300 μg protein/ml. After 2 min of equilibration, aliquots of the incubation mixture were withdrawn in duplicate (t = 0 min), and the vesicles were sedimented in microfuge
tubes at 7,000 g for 45 s. Total Mg$^{2+}$ content in the supernatants was measured by AAS. The residual Mg$^{2+}$ content within the vesicles was also measured by AAS after overnight digestion of the vesicle pellets in 500 μl of 10% HNO$_3$ and sedimentation of denatured proteins at 7,000 g for 5 min in microfuge tubes. The first time point after the equilibration period (t = 0) was used to assess basal vesicular and extravesicular Mg$^{2+}$ level. After the withdrawal of the sample, Mg$^{2+}$ transport was stimulated by addition of 25 mM Na$^+$ to the incubation mixture. The incubation was continued for 6 additional minutes, and samples were withdrawn in duplicate at 2-min intervals. Because Mg$^{2+}$ content in the supernatant could vary considerably among preparations as a result of the loading procedure and carry-over, the data are reported as the net variation in extravesicular (or vesicular) Mg$^{2+}$ content, normalized per milligram of protein for simplicity. To determine net Mg$^{2+}$ extrusion, Mg$^{2+}$ content in the supernatant at t = 0 min was calculated and subtracted from the values of the subsequent time points of incubation.

Similar experimental procedures were used for Na$^+$-loaded, Mg$^{2+}$-stimulated vesicles.

Statistical analysis. The data are reported as means ± SE. Data were first analyzed by one-way ANOVA. Multiple means were then compared by Tukey’s multiple comparison test performed with a q value established for statistical significance of P < 0.05.

RESULTS

Hepatic cation content. Rats rendered diabetic by streptozotocin injection presented an ~30% decrease in body weight compared with age-paired controls (256 ± 12 vs. 377 ± 18 g, respectively; n = 10 for each group) after 4 wk from the induction of diabetes. The decrease in body weight progressed with time and became more marked (~40%) at 8 wk from diabetes onset. Four-week diabetic animals presented a four- to fivefold increase in glyceremia compared with age-matched controls (435 ± 25 vs. 85 ± 5 mg/100 ml, respectively). As shown in Table 1, total Mg$^{2+}$ content decreased by ~10% in liver of rats diabetic for 4 wk. Potassium content also decreased to a comparable extent, whereas Na$^+$ content increased. Calcium content also appeared to increase, although the amplitude of the changes was smaller compared with that of Na$^+$, Mg$^{2+}$, or K$^+$ (not shown) and did not reach statistical significance. The changes in tissue cation content progressed over time. At 8 wk after diabetes induction, liver Mg$^{2+}$ and K$^+$ content were decreased by ~20% compared with age-matched nondiabetic rats, whereas tissue Na$^+$ content increased proportionally (Table 1). Liver Mg$^{2+}$ and K$^+$ content were similarly lower compared with the levels determined in livers of weight-matched nondiabetic rats (not shown), thus excluding that the observed changes depended on difference in development. Streptozotocin-injected animals that did not develop diabetes presented hepatic cation content similar to that of nondiabetic animals (not shown).

Mg$^{2+}$ mobilization. In the past decade, our laboratory (9, 10, 27–31) has steadily investigated the hormonal mechanisms that control cellular Mg$^{2+}$ homeostasis in liver cells. Hence, we used perfused livers and collagenase-dispersed hepatocytes to assess whether 1) alteration in transmembrane Mg$^{2+}$ transport could be responsible for the depletion in hepatic Mg$^{2+}$ content, and 2) an accumulation of Mg$^{2+}$ into liver cells could be induced to restore cellular cation homeostasis.

Table 1. Total liver cation content in 4- and 8-wk diabetic and age-matched nondiabetic animals

<table>
<thead>
<tr>
<th></th>
<th>Na$^+$</th>
<th>K$^+$</th>
<th>Mg$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nondiabetic</td>
<td>Diabetic</td>
<td>Nondiabetic</td>
</tr>
<tr>
<td>4 wk</td>
<td>7.9±0.9</td>
<td>9.6±0.6*</td>
<td>68.1±4.6</td>
</tr>
<tr>
<td>8 wk</td>
<td>8.3±0.3</td>
<td>10.8±0.8*</td>
<td>67.8±3.4</td>
</tr>
</tbody>
</table>

Data are means ± SE of 12 animals for each experimental group. Cation contents are represented as μmol/g wet tissue. *Statistically significant vs. nondiabetic value.
experimental conditions, the infusion of isoproterenol resulted in a marked extrusion of Mg\(^{2+}\) into the perfusate, which was quantitatively similar in diabetic and nondiabetic livers (1.7 vs. 1.5 μmol, respectively, as total net Mg\(^{2+}\) extrusion). Similar results were also obtained in livers stimulated by 250 μM 8-Cl-cAMP (a cell-permeant cAMP analog) instead of isoproterenol (not shown). The stimulation of α1-adrenoceptor by phenylephrine instead elicited a sizable extrusion of Mg\(^{2+}\) from nondiabetic livers but not from livers of diabetic rats, in which it was ~70% reduced (~3.75 vs. ~1.1 μmol, respectively; Figs. 1B and 2A). The estimate of the total amount of Mg\(^{2+}\) mobilized from the liver into the perfusate, calculated as reported in MATERIALS AND METHODS, confirmed a reduced responsiveness of α1-adrenoceptor signaling in diabetic livers, while indicating that phenylephrine mobilizes a larger amount of Mg\(^{2+}\) than isoproterenol in nondiabetic livers (~3.75 and ~1.50 μmol, respectively; Fig. 2A). Similar results were obtained in livers stimulated by methoxamine as α1-adrenoceptor agonist (data not shown). As expected, a negligible amount of glucose (<5 μmol/ml) was mobilized from diabetic livers by α1- or β-adrenergic receptor stimulation compared with ~30 μmol/ml glucose extruded by either agonist in nondiabetic livers (Fig. 2B). The administration of isoproterenol and phenylephrine elicited similar results in livers from 8-wk diabetic rats (Figs. 3, A and B, respectively). The profile of Mg\(^{2+}\) extrusion induced by the mixed adrenergic agonist epinephrine was also markedly different in diabetic compared with nondiabetic livers (Fig. 3C). In both nondiabetic and diabetic livers,

Fig. 2. Net Mg\(^{2+}\) and glucose extrusion from 4-wk-perfused rat livers. The net amount of Mg\(^{2+}\) (A) and glucose (B) extruded from rat livers reported in Fig. 1 were estimated as described in MATERIALS AND METHODS. Data are means ± SE of 6 different preparations for each experimental condition. *Statistically significant vs. value in nondiabetic animals.

Fig. 3. Mg\(^{2+}\) extrusion from perfused livers stimulated by β- and α1-adrenergic agonist. Livers from 8-wk diabetic and age-matched nondiabetic rats were perfused as described in MATERIALS AND METHODS. After a few minutes of equilibration, 10 μM isoproterenol (A), 5 μM phenylephrine (B), or 5 μM epinephrine (C) were dissolved directly into the perfusion medium and administered for 10 min. Data are means ± SE of 6 different preparations for each experimental condition. *All data points under the curve of efflux in B and C were statistically significant vs. corresponding values in nondiabetic animals. Labeling omitted for simplicity.
epinephrine elicited a Mg$^{2+}$ extrusion that appeared to be quantitatively equivalent to the amounts of Mg$^{2+}$ mobilized separately by phenylephrine and isoproterenol, as reported previously (9). In diabetic livers, however, epinephrine infusion resulted in a smaller and transient increase in Mg$^{2+}$ extrusion, which returned toward basal level within 4 min from the agonist administration. At later time points following agonist removal, Mg$^{2+}$ baseline appeared to be slightly elevated compared with nonstimulated livers and with nondiabetic livers treated with epinephrine (Fig. 3C); yet, the difference did not achieve statistical significance, nor was it accompanied by release of cellular LDH or K$^+$. The Mg$^{2+}$ extruded at these later time points was not taken into account in estimating total Mg$^{2+}$ extrusion under these conditions, and the phenomenon was not further investigated at the present time. Glucose output from livers of 8-wk diabetic animals remained considerably lower than the values measured in age-matched nondiabetic rats (not shown).

To better quantitate the amplitude of Mg$^{2+}$ extrusion and to determine whether the loss of cellular Mg$^{2+}$ resulted in a particular redistribution of Mg$^{2+}$ within the cell, collagenase-dispersed hepatocytes were used under in vitro conditions. Hepatocytes isolated from 4-wk diabetic rats presented a 25% decrease in total Mg$^{2+}$ content compared with age-paired controls (26.0 ± 0.6 vs. 36.1 ± 0.8 nmol/mg protein, respectively) and an ~20% decrease compared with liver cells from weight-matched nondiabetic animals (34.7 ± 0.5 nmol/mg protein; n = 4). When determined in hepatocytes from 8-wk diabetic rats, total Mg$^{2+}$ content was further reduced vs. age-matched controls (24.2 ± 0.5 vs. 36.9 ± 0.4 nmol/mg protein, or minus ~35%). The decrease in cellular Mg$^{2+}$ content was associated with a 17% decline in total ATP content at 4 wk after diabetes onset (~3.2 vs. 4.1 nmol ATP/mg protein in diabetic vs. nondiabetic hepatocytes, respectively). This decline was essentially similar to that determined in total liver homogenate of 4-wk diabetic rats compared with age-matched nondiabetic animals (11.5 ± 0.1 vs. 14.1 ± 0.3 nmol ATP/mg protein, respectively; n = 9). A similar decline was also observed in liver cells from 8-wk diabetic rats vs. their nondiabetic controls (3.0 ± 0.2 vs. 4.1 ± 0.3 nmol ATP/mg protein, respectively). The decline in cellular Mg$^{2+}$ and ATP content directly correlated at both 4 wk (r = 0.999, P < 0.0001) and 8 wk (r = 0.998, P < 0.01). Similar correlation values were also obtained when the declines in ATP and Mg$^{2+}$ were correlated as percent changes. The decrease in Mg$^{2+}$ content appeared to affect to a varying extent all cellular compartments (Fig. 4). The sequential administration of digitonin, mitochondria uncoupler FCCP, and ionophore A-23187 indicated a 20% decrease in cytosolic Mg$^{2+}$ (digitonin), a 35% decrease in mitochondrial Mg$^{2+}$ (FCCP), and a ~50% decrease in postmitochondrial Mg$^{2+}$ (A-23187) in hepatocytes from 4-wk diabetic rats compared with age-matched nondiabetic animals. Figure 5A illustrates the incubation protocol utilized to assess Mg$^{2+}$ transport in suspensions of hepatocytes from 4-wk diabetic and nondiabetic rats. In the absence of any stimulatory agent, hepatocytes in suspension did not release a detectable amount of Mg$^{2+}$ into the extracellular space. After the addition of adrenergic agonist, the cells extruded a significant amount of Mg$^{2+}$ into the external compartment. The net
amount of Mg\(^{2+}\) mobilized by phenylephrine or isoproterenol from nondiabetic hepatocytes accounted for 1.6 ± 0.4 and 1.2 ± 0.3 nmol Mg\(^{2+}\)/mg protein, respectively, over 4 min of stimulation (Fig. 5B). Under similar experimental conditions, diabetic hepatocytes mobilized 0.2 ± 0.04 and 1.1 ± 0.2 nmol Mg\(^{2+}\)/mg protein, respectively (Fig. 5B). Also in this experimental model, epinephrine elicited a Mg\(^{2+}\) extrusion that was quantitatively equivalent to the amounts mobilized separately by isoproterenol and phenylephrine, although it remained significantly smaller in diabetic compared with nondiabetic hepatocytes (1.8 ± 0.3 vs. 3.5 ± 0.5 nmol Mg\(^{2+}\)/mg protein \(^{-1}\)·6 min \(^{-1}\), respectively; \(n = 8\), \(P < 0.05\)). The inability of phenylephrine (or methoxamine, not shown) to mobilize Mg\(^{2+}\) from diabetic hepatocytes cannot be ascribed merely to a defect in receptor responsiveness, as a similar lack of extrusion was observed in hepatocytes treated with thapsigargin, an agent that mimics \(\alpha_1\)-adrenoceptor-mediated Mg\(^{2+}\) extrusion in liver cells bypassing the receptor (10). The addition of 2 \(\mu\)M thapsigargin, in fact, elicited the extrusion of 0.4 ± 0.1 nmol Mg\(^{2+}\)/mg protein \(^{-1}\)·6 min \(^{-1}\) from diabetic hepatocytes compared with 1.7 ± 0.2 nmol Mg\(^{2+}\)/mg protein \(^{-1}\)·6 min \(^{-1}\) from nondiabetic cells (\(n = 8\), \(P < 0.05\)). Cellular Mg\(^{2+}\) partitioning in nondiabetic hepatocytes undergoing stimulation by adrenergic agonists indicated a decrease at the cytoplasmic and mitochondrial levels following stimulation by isoproterenol (−7 and −37%, respectively, vs. basal values) and a decrease in the postmitochondrial pool (−21% vs. basal value) after phenylephrine stimulation (Fig. 6). In hepatocytes from diabetic rats instead, isoproterenol stimulation resulted in a further decrease in cytoplasm and mitochondria pools compared with the level before agonist addition (−6 and −18%, respectively), whereas phenylephrine addition did not elicit an appreciable decrease in the postmitochondrial pool (Fig. 6). When correlated with the basal Mg\(^{2+}\) content present in the different cellular compartments, the amount of Mg\(^{2+}\) extruded by isoproterenol or phenylephrine stimulation in nondiabetic hepatocytes resulted in the values \(r = 0.177\), \(P < 0.01\) and \(r = 0.133\), \(P < 0.02\), respectively, for the cytosol; \(r = -0.948\), \(P < 0.01\) and \(r = 1.55e^{-15}\), \(P < 0.001\), respectively, for mitochondria, and \(r = 0.755\), \(P < 0.1\) and \(r = -0.07\), \(P < 0.1\), respectively, for the postmitochondrial pools. In diabetic hepatocytes, similar correlation analysis resulted in values of \(r = 0.42\), \(P < 0.06\) and \(r = -0.73\), \(P < 0.03\), respectively, for the cytosol; \(r = 0.24\), \(P < 0.01\) and \(r = -0.91\), \(P < 0.01\), respectively, for mitochondria, and \(r = -0.53\), \(P < 0.03\) and \(r = 0.98\), \(P < 0.01\), respectively, for the postmitochondrial compartments. Comparable values were obtained when basal Mg\(^{2+}\) content was correlated with percent magnesium extrusion following \(\beta\)- and \(\alpha_1\)-adrenoceptor agonist stimulation. The correlation between the Mg\(^{2+}\) content present within the various cellular compartments before agonist stimulation and the Mg\(^{2+}\) content remaining therein after adrenergic stimulation resulted in the values \(r = 0.994\), \(P < 0.002\) and \(r = 0.998\), \(P < 0.001\) for isoproterenol- and phenylephrine-stimulated nondiabetic hepatocytes, respectively, and \(r = 0.993\), \(P < 0.002\) and \(r = 0.998\), \(P < 0.001\), respectively, in diabetic hepatocytes. Finally, the correlation between the amount of Mg\(^{2+}\) lost from the different compartments and the net amount of Mg\(^{2+}\) mobilized into the extracellular compartment resulted in the values \(r = 0.30\), \(P < 0.02\) and \(r = 0.23\), \(P < 0.01\) for isoproterenol- and phenylephrine-stimulated nondiabetic cells, respectively. In diabetic hepatocytes, inverse correlations were measured for both isoproterenol (\(r = -0.59\), \(P < 0.01\) and phenylephrine stimulation (\(r = -0.10\), \(P < 0.01\)).

The experimental protocol reported in Fig. 5A was also used to investigate whether hepatocytes from 4-wk diabetic animals could accumulate Mg\(^{2+}\) following stimulation of the protein kinase C-signaling pathway. After 3 min of equilibration, various agents that stimulate Mg\(^{2+}\) accumulation in liver cells were added to the incubation mixture. As Fig. 7A indicates, the administration of vasopressin, insulin, oleoyl-arachidonoyl glycerol (OAG, a cell-permeant diacylglycerol analog), or phorbol 12,13-dibutyrate (PDBu, an analog of PMA), all induced a net accumulation of \(\sim 1-1.5\) nmol Mg\(^{2+}\)/mg protein \(^{-1}\)·4 min \(^{-1}\) in hepatocytes from nondiabetic animals. In contrast, hepatocytes from diabetic animals were unable to accumulate Mg\(^{2+}\) irrespective of the agonist (Fig. 7A), the dose of the agent (not shown), or the extracellular Mg\(^{2+}\) concentration (Fig. 7B) utilized. In nondiabetic hepatocytes, the amplitude of Mg\(^{2+}\) accumulation elicited by the various protein kinase C agonists was unaffected by changes in extracellular Mg\(^{2+}\) concentration (not shown; see Ref. 29). Qualitatively similar results in terms of Mg\(^{2+}\) accumulation and extrusion were also observed in hepatocytes isolated from 8-wk diabetic animals (not shown).

We (5) have evidenced the operation of bidirectional Na\(^{+}\)−dependent Mg\(^{2+}\) transport mechanisms in purified tLPM vesicles. To determine whether the lack of Mg\(^{2+}\) accumulation depended on the defective operation of the Mg\(^{2+}\) entry mechanisms, tLPM vesicles were purified from 4-wk diabetic and age-matched nondiabetic rats and loaded with 20 mM Mg\(^{2+}\) or 20 mM Na\(^{+}\). As Fig. 8A indicates, Mg\(^{2+}\)-loaded diabetic tLPM vesicles extruded about three times more Mg\(^{2+}\) than tLPM from nondiabetic livers following addition of 25 mM Na\(^{+}\) in the extravesicular space. In contrast, when the cation gradient was reversed, diabetic tLPM did not accumulate

Fig. 6. Net Mg\(^{2+}\) extrusion from various intracellular compartments. Hepatocytes from 4-wk diabetic and age-matched nondiabetic rats were isolated and incubated as indicated in MATERIALS AND METHODS. After a few minutes of equilibration, cells were stimulated with 10 \(\mu\)M isoproterenol or 5 \(\mu\)M phenylephrine for 6 min. Digitonin (50 \(\mu\)g/ml), FCCP (52 \(\mu\)g/ml), and A-23187 (2 \(\mu\)g/ml) were then added sequentially at 5-min intervals. Net Mg\(^{2+}\) extrusion was calculated as described in MATERIALS AND METHODS using the values at 6 min as basal level. Data are means ± SE of 6 different preparations, each performed in quadruplicate. All values for diabetic hepatocytes are statistically significant vs. nondiabetic hepatocytes. Labeling omitted for simplicity.
As indicated in the introductory section, experimental and clinical evidence indicates that plasma and tissue Mg\(^{2+}\) contents are markedly decreased in both type 1 and type 2 diabetes (26, 40). Although a decreased phosphorylation of insulin receptor and downstream signaling effectors has been observed in Mg\(^{2+}\)-depleted tissues of animals fed a Mg\(^{2+}\)-deficient diet (35), no organic study has been carried out to date to elucidate the modality by which Mg\(^{2+}\) loss occurs in diabetic animals or patients and the short- and long-term implications of Mg\(^{2+}\) deficiency for the proper functioning of cellular metabolic processes under this pathological condition.

The present study was undertaken to address some of these questions. In particular, we wanted to investigate whether the decrease in Mg\(^{2+}\) content could be ascribed to an altered transport across the cell membrane of the hepatocyte.

Mg\(^{2+}\) homeostasis within the hepatocyte. The onset of type 1 diabetes results in a time-dependent loss of hepatic Mg\(^{2+}\), which is associated with a comparable loss of K\(^{+}\) and ATP and an increase in Na\(^{+}\) content. The increase in tissue Na\(^{+}\) content (and Ca\(^{2+}\); not shown) excludes the possibility that Mg\(^{2+}\) and K\(^{+}\) loss is a nonspecific process. Although the decreased activity of Na\(^{-}\)-K\(^{+}\)-ATPase observed in tLPM and homoge-

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**Fig. 7.** Mg\(^{2+}\) accumulation in isolated hepatocytes. Hepatocytes from 4-wk diabetic and age-matched nondiabetic rats were incubated in a medium containing trace contaminant (A) or 1 mM external Mg\(^{2+}\) (B). After a few minutes of equilibration, the hepatocytes were stimulated by addition of vasopressin (AVP, 20 nM), insulin (10 nM), oleoyl-arachidonoyl glycerol (OAG, 20 nM), and phorbol 12,13-dibutyrate (PDBu, 20 μM). After 4 min of stimulation, total Mg\(^{2+}\) content in the cell pellet was determined as described in MATERIALS AND METHODS. Data are means ± SE of 6 different experiments for all experimental conditions, each performed in quadruplicate. A: *statistically significant vs. nondiabetic hepatocytes. B: all data points statistically significant vs. nondiabetic hepatocytes values reported in A. Labeling omitted for simplicity.

Mg\(^{2+}\) in exchange for vesicular entrapped Na\(^{+}\) (Fig. 8B). Under similar experimental conditions, nondiabetic tLPM accumulate ~200 nmol Mg\(^{2+}\)/mg protein within 2 min from the addition of 20 mM extravesicular Mg\(^{2+}\) (Fig. 8B).

Streptozotocin-injected animals that did not develop diabetes were used as additional controls throughout the study. Liver cells and tLPM from these animals extruded and accumulated Mg\(^{2+}\) in a manner similar to nondiabetic animals (not shown), excluding therefore that the observed effects in perfused livers, isolated cells, or tLPM were due to drug metabolism.

**DISCUSSION**

The past decade has registered an increasing interest in understanding how Mg\(^{2+}\) is transported and regulated in mammalian cells as well as the implications that changes in cellular Mg\(^{2+}\) content have for the overall cell functioning under physiological and pathological conditions.

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**Fig. 8.** Mg\(^{2+}\) transport in total liver plasma membranes (tLPM). tLPM from 4-wk diabetic and nondiabetic rats were isolated and loaded with 20 mM Mg\(^{2+}\) (A) or 20 mM Na\(^{+}\) (B). Mg\(^{2+}\) extrusion was elicited by extravesicular addition of 25 mM Na\(^{+}\) (A). Magnesium accumulation was elicited by addition of 20 mM Mg\(^{2+}\) in the extravesicular space (B). *Statistically significant vs. corresponding data point in nondiabetic tLPM.
nate under diabetic conditions can explain the modifications in Na\(^+\) and K\(^+\) content, other mechanisms have to be invoked to explain the decrease in hepatic Mg\(^ {2+}\) content.

The larger loss in Mg\(^ {2+}\) content detected in hepatocytes compared with total liver extract is probably due to the removal of collagen and tissue components other than the hepatocytes, which introduces some degree of inaccuracy in total tissue determination. Within the cell, Mg\(^ {2+}\) loss appears to affect all organelles and compartments, including cytoplasm. In fact, the depletion in cytosolic Mg\(^ {2+}\) (~20%; Fig. 4) directly correlates with and is almost superimposable on the 17% decrease in ATP content, further supporting the notion that ATP represents the main regulatory component of cytosolic Mg\(^ {2+}\) content (33). Based on a total ATP content of 4 mM, a 17% decrease corresponds to ~700 \(\mu\)M. As ATP is degraded to ADP and AMP, the dissociation constant of these moieties for Mg\(^ {2+}\) decreases from ~80 \(\mu\)M (Mg-ATP) to ~8.13 mM (Mg-AMP), resulting in the dissociation of a significant amount of Mg\(^ {2+}\) that ultimately is extruded from the cell. The discrepancy between the total amount of cellular Mg\(^ {2+}\) (10 nmol/mg protein) vs. ATP content (~1 nmol/mg protein) lost from the diabetic hepatocytes can be explained by the fact that Mg\(^ {2+}\) is lost from all cellular organelles and compartments (in particular the postmitochondrial compartments) and not merely from the cytoplasm and mitochondria, in which ATP is predominantly located. Although phosphocreatine was not measured in the present study, a decrease in ATP content of the amplitude observed here (~17%) would imply a decrease in this ATP-regenerating moiety. Because phosphocreatine binds cytoplasmic Mg\(^ {2+}\) as well (30), its decrease is likely to contribute to the decrease in cytoplasmic Mg\(^ {2+}\) observed in diabetic hepatocytes. A similar decrease in Mg\(^ {2+}\) and ATP content has been observed in hepatocytes treated acutely (38) or chronically (43) with EtOH. This result suggests that the cell possesses a specific but still-identified mechanism that senses variations in cytosolic Mg\(^ {2+}\) and determines the extrusion of excess Mg\(^ {2+}\) across the plasma membrane. As for the changes in other cellular compartments, they can be either secondary to the changes in bound/free Mg\(^ {2+}\) in the cytoplasm or consequence to a direct modification of the organelle functioning under diabetic conditions. It has been reported that Mg\(^ {2+}\) plays a key role in regulating various organelle enzymes, including mitochondrial dehydrogenases (23) and reticular ATPases (8). Hence, it can be speculated that a loss of Mg\(^ {2+}\) within organelles can contribute to some of the metabolic changes observed under diabetic conditions. This speculation is supported by the correlation between Mg\(^ {2+}\) content within cellular organelles and the amplitude of Mg\(^ {2+}\) extrusion following \(\beta\)-and \(\alpha_1\)-adrenergic stimulation in nondiabetic and diabetic hepatocytes.

**Mg\(^ {2+}\) entry across the cell membrane.** The most important information provided by this study is that liver cells from diabetic animals are unable to accumulate Mg\(^ {2+}\) to restore the cation cellular homeostasis. This defect persists irrespective of the extracellular Mg\(^ {2+}\) concentration (contaminant or physiological 1 mM) and is consistent with the hypothesis that Mg\(^ {2+}\) entry does not depend on the Mg\(^ {2+}\) gradient across the cell membrane but is a tightly regulated process (14, 32). Previous reports indicate that Mg\(^ {2+}\) accumulation in liver cell is regulated via protein kinase C activation (28) and inhibited under conditions in which protein kinase C is downregulated (28) or intracellular Ca\(^ {2+}\) is elevated (30). Under diabetic conditions, defects in protein kinase C signaling (37) and an altered Ca\(^ {2+}\) cycling between cytoplasm and sarcoplasmic reticulum (18) have both been reported and attributed to changes in the plasma membrane phospholipid environment (22), an altered cross talk among signaling molecules (11), or an abnormal operation of the reticular Ca\(^ {2+}\)-sequestering/releasing mechanisms (18). The inability of diabetic tLPM to accumulate Mg\(^ {2+}\) in exchange for intravesicular Na\(^ +\) (Fig. 8B) via the putative bidirectional Na\(^ +\)/Mg\(^ {2+}\) exchanger (5) indicates that functional (e.g., faulty signaling or phosphorylation) and/or structural modifications (e.g., glycation at the extracellular side or alteration in the phospholipid environment) affect directly or indirectly the Mg\(^ {2+}\) transport mechanism. Further studies are necessary to discriminate among these possibilities. The possibility that Mg\(^ {2+}\) entry is an energy-dependent process (e.g., a pump) that becomes defective in diabetic hepatocytes due to the reduced cellular ATP level appears to be unlikely, as results obtained in nondiabetic tLPM indicate that Mg\(^ {2+}\) enters the vesicle in exchange for entrapped Na\(^ +\) in the absence of intravesicular ATP (Fig. 8B; see also Ref. 5). Yet, a reduced phosphorylation/activation of the Mg\(^ {2+}\) transporter as a consequence of the reduced cellular level of ATP cannot be excluded altogether.

**Mg\(^ {2+}\) extrusion across the cell membrane.** Our results also indicate that a difference exists between \(\alpha_1\)- and \(\beta\)-adrenoceptor-mediated Mg\(^ {2+}\) extrusion processes. Although the latter process appears to operate normally irrespective of the time elapsed since diabetes onset, the \(\alpha_1\)-adrenergic receptor-mediated process is markedly reduced (~50% or more). As both classes of adrenoceptor activate the same Mg\(^ {2+}\) extrusion mechanism [i.e., the Na\(^ +\)/Mg\(^ {2+}\) exchanger (9, 10)] that appears to be operative in cells stimulated by \(\beta\)-adrenoceptor agonist and tLPM (Fig. 7A), we have to hypothesize that the defect is at the level of 1) the \(\alpha_1\)-adrenoceptor, 2) the associated signaling pathway activating the transporter, or 3) the cellular compartment from which Mg\(^ {2+}\) should be mobilized. Although the first two possibilities are not excluded, the inability of thapsigargin to restore Mg\(^ {2+}\) extrusion bypassing the \(\alpha_1\)-adrenoceptor (10) suggests that the depletion of the cellular Mg\(^ {2+}\) compartment targeted by \(\alpha_1\)-adrenoceptor stimulation (most likely the endoplasmic reticulum) is one of the main reasons for the lack of Mg\(^ {2+}\) extrusion under these conditions. Further support for this possibility is provided by the estimate of cellular Mg\(^ {2+}\) compartmentalization (Fig. 4), which indicates a ~50% depletion of postmitochondrial pools under diabetic conditions, which does not change significantly following phenylephrine stimulation (Fig. 6). In contrast, administration of isoproterenol to diabetic hepatocytes results in a further decrease in cytoplasmic and mitochondrial Mg\(^ {2+}\) content compared with basal level (Fig. 6). These results, however, do not necessarily exclude the occurrence of Mg\(^ {2+}\) redistribution among cellular compartments to an extent that may vary in diabetic compared with nondiabetic cells. Additional, albeit indirect, evidence for the mobilization of Mg\(^ {2+}\) from distinct cellular pools following \(\alpha_1\)- and \(\beta\)-adrenoceptor stimulation (9) is provided by the normal response to \(\beta\)-adrenoceptor agonist (isoproterenol) or its second messenger cAMP and by the partial responsiveness of diabetic liver cells to the mixed adrenoceptor agonist epinephrine. As mitochondrial and cytosolic Mg\(^ {2+}\) pools appear to be less depleted than...
postmitochondrial (ionophore-sensitive) pools, it can be speculated that they constitute the intracellular pool(s) involved in β-adrenergic-mediated Mg\(^{2+}\) extrusion. This hypothesis will be consistent with an early report from this laboratory (27) about the occurrence of a cAMP-mediated Mg\(^{2+}\) extrusion from permeabilized hepatocytes or isolated liver mitochondria. It must be noted that the normal Mg\(^{2+}\) extrusion elicited via β-adrenergic receptor activation in liver cells appears to contrast with data available in the literature indicating a defective \(\alpha_1\)-adrenergic receptor activation in liver cells appears to contribute to explain it.

Overall, the comparison between the percent and absolute decrease in cellular and intracellular ion content (−35 to −45%, according to the compartment considered), and the decreased amplitude of Mg\(^{2+}\) extrusion by phenylephrine or mixed adrenergic agonist (~50%) or uptake following OAG or PMA stimulation observed in diabetic hepatocytes suggests a reduced steady state in cellular Mg\(^{2+}\) as a result of a reduced buffering capacity and intracellular storing. This would be consistent with a general property of ion homeostasis, by which cellular steady-state level regulates cellular responsiveness to extracellular and intracellular mobilizing stimuli (1, 2). However, caution must be exercised in interpreting our data, in that the high concentration of glucose utilized under our experimental conditions, which is two- to threefold larger than intracellular ion concentration, may perturb intracellular ion buffering capacity, and may alter intracellular ion concentration (1, 3).

In conclusion, the results of our study indicate that liver cells from type 1 diabetic rats present a considerable loss of Mg\(^{2+}\). This Mg\(^{2+}\) loss depends on a decrease in cellular ATP and the inability of the hepatocyte to accumulate Mg\(^{2+}\) from the extracellular compartment and restore cellular Mg\(^{2+}\) homeostasis. The deficit is localized at the level of the Mg\(^{2+}\) entry mechanism in the plasma membrane and possibly on its activating signaling. Overall, these results provide a basic understanding of the defect(s) responsible for the decrease in cellular Mg\(^{2+}\) content under diabetic conditions as well as providing an important framework for further studies aimed at determining the role this Mg\(^{2+}\) loss plays for the short- and long-term complications of diabetes.

GRANTS

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