Journal of Cellular Biochemistry 104:1034–1053 (2008)

Lack of Insulin Impairs Mg^{2+} Homeostasis and Transport in Cardiac Cells of Streptozotocin-Injected Diabetic Rats

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Abstract Serum and tissue Mg^{2+} content are markedly decreased in diabetic patients and animals. At the tissue level, Mg^{2+} loss progresses over time and affects predominantly heart, liver and skeletal muscles. In the present study, alterations in Mg^{2+} homeostasis and transport in diabetic cardiac ventricular myocytes were evaluated. Cardiac tissue and isolated cardiac ventricular myocytes from diabetic animals displayed a decrease in total Mg^{2+} content that affected all cellular compartments. This decrease was associated with a marked reduction in cellular protein and ATP content. Diabetic ventricular myocytes were unable to mobilize Mg^{2+} following β-adrenergic receptor stimulation or addition of cell permeant cyclic-AMP. Sarcolemma vesicles purified from diabetic animals, however, transported Mg^{2+} normally as compared to vesicles from non-diabetic animals. Treatment of diabetic animals with exogenous insulin for 2 weeks restored ATP and protein levels as well as Mg^{2+} homeostasis and transport to levels comparable to those observed in non-diabetic animals. These results suggest that in diabetic cardiac cells Mg^{2+} homeostasis and extrusion via β-adrenergic/cAMP signaling are markedly affected by the concomitant decrease in protein and ATP content. As Mg^{2+} regulates numerous cellular enzymes and functions, including protein synthesis, these results provide a new rationale to interpret some aspects of the cardiac dysfunctions observed under diabetic conditions. J. Cell. Biochem. 104: 1034–1053, 2008.

Key words: insulin; Mg^{2+} distribution; Mg^{2+} extrusion; type-I diabetes; adrenergic signaling; cardiac cells

Magnesium (Mg^{2+}) is the second most abundant cation within mammalian cells after potassium [Gunther, 1986; Romani and Scarpa, 2000]. Mapping by electron probe X-rays microanalysis (EPXMA[1]) indicates the mitochondria, endo-sarco-plasmic reticulum and nucleus as the three main intracellular Mg^{2+} pools, with concentrations ranging between 16 and 20 mM in each of these compartments [Gunther, 1986; Romani and Scarpa, 2000; Wolf et al., 2003]. Cytoplasm also contains a sizable amount of Mg^{2+} (4–5 mM), mostly in the form of a complex with ATP, phosphocreatine and other phosphonucleotides [Scarpa and Brinley, 1981; Romani and Scarpa, 2000]. As a result of this distribution, in the majority of mammalian cells considered including cardiac myocytes cytoplasmic free Mg^{2+} concentration ($[Mg^{2+}]_i$) ranges between 0.5 and 0.7 mM [Gunther, 1986; Romani and Scarpa, 2000; Wolf et al., 2003], a concentration close to that present outside the cell or free within the mitochondrial matrix [Gunther, 1986; Jung and Brierley, 1999; Romani and Scarpa, 2000; Wolf et al., 2003].

Under physiological conditions, no significant variations in total and free cellular Mg^{2+} concentrations have been observed [Gunther, 1986; Romani and Scarpa, 2000; Wolf et al., 2003]. The application of specific hormonal stimuli, however, results in a major Mg^{2+} efflux across the cell membrane. This efflux results in a detectable decrease in total cellular Mg^{2+} content whereas $[Mg^{2+}]_i$ varies only minimally [Fatholahi et al., 2000]. The

Abbreviations used: EPXMA, electron probe X-rays microanalysis; FCCP, carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone; ISO, isoproterenol; EPI, epinephrine; 8-Cl-cAMP, 8-Chloro-phenyl-thio-cyclic AMP; SL, sarcolemmal vesicles.

Grant sponsor: NIH; Grant number: HL18708; Grant sponsor: NIH-AAA; Grant number: AA11593.

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Received 3 November 2007; Accepted 10 December 2007
DOI 10.1002/jcb.21690

hormone-induced Mg^{2+} extrusion was first observed in perfused rat hearts [Vormann and Gunther, 1987; Romani and Scarpa, 1990a] and livers [Romani and Scarpa, 1990b]. Using a variety of experimental or pharmacological tools, subsequent studies elucidate the signaling involved (i.e., β-adrenergic receptor and cAMP) [Romani and Scarpa, 1990a, 1990b] and the dependence of Mg^{2+} extrusion on extracellular Na^+ [Gunther and Vormann, 1985; Romani et al., 1993] All these studies have confirmed that: (1) the mechanism originally described in cardiac cells [Vormann and Gunther, 1987; Romani and Scarpa, 1990a] and hepatocytes [Romani and Scarpa, 1990b] is ubiquitous as its operation has been reported in erythrocytes [Matsuura et al., 1993], lymphocytes [Wolf et al., 1997], and almost all the cells tested [reviewed in Romani and Scarpa, 2000]; and (2) cAMP is essential to activate the Mg^{2+} extrusion mechanism, most likely via phosphorylation [Gunther and Vormann, 1992], irrespective of the agent or hormone utilized to increase cellular cAMP level. Activation of β-adrenergic [Vormann and Gunther, 1987; Romani and Scarpa, 1990a, 1990b], glucagon [Fagan and Romani, 2000] or PGE2 receptors [Wolf et al., 1997], administration of cell-permeant cAMP analogs (e.g., 8-Cl-cAMP or dibutyryl-cAMP) [Vormann and Gunther, 1987; Romani and Scarpa, 1990a,b], or forskolin [Romani and Scarpa, 1990a,b], all elicit Mg^{2+} extrusion in perfused organs [Vormann and Gunther, 1987; Romani and Scarpa, 1990a,b; Fagan and Romani, 2000], isolated cells [Romani and Scarpa, 1990a,b; Fagan and Romani, 2000], and the whole animal [Keenan et al., 1995], supporting the ubiquitous nature of the process. Consistent with a key role of cAMP in eliciting Mg^{2+} extrusion, agents (e.g., Rp-cAMP isomer [Wolf et al., 1997]) or hormones [Keenan et al., 1996] that inhibit adenylyl cyclase or prevent its activation all prevent cAMP-mediated Mg^{2+} extrusion.

Under the majority of these experimental conditions, Mg^{2+} is extruded through the cell membrane via a mechanism tentatively described as a Na^+/Mg^{2+} exchanger [Flatman, 1991]. As this transporter has not yet been cloned, its operation is largely inferred by the observation that Mg^{2+} extrusion is inhibited following removal of extracellular Na^+ or administration of non-selective Na^+-transport inhibitors such as amiloride, imipramine or quinidine [Gunther and Vormann, 1985; Feray and Garay, 1988; Romani et al., 1993].

Despite the relevance of Mg^{2+} content for various cellular functions, limited and mostly anecdotal information is available about defect in cellular Mg^{2+} transport and homeostasis under pathological conditions. Experimental and clinical evidence indicates a loss of tissue and plasma Mg^{2+} under type-I and type-II diabetes [Wallach and Verch, 1987; Resnick et al., 1993]. However, neither the mechanism by which Mg^{2+} is lost from the tissue nor the contribution of this Mg^{2+} loss to the development of diabetic complications have been elucidated.

Our laboratory has reported that induction of diabetes by streptozotocin injection results in a progressive loss of Mg^{2+} from liver cells [Fagan et al., 2004]. In spite of this Mg^{2+} loss, diabetic hepatocytes retained the ability to extrude Mg^{2+} following β-adrenergic stimulation [Fagan et al., 2004].

A deficit in β-adrenergic receptor stimulation and G-protein signaling in diabetic hearts has been reported [Roth et al., 1995]. Hence, the present study was undertaken to assess whether: (1) Mg^{2+} loss also occurs in cardiac cells from streptozotocin-injected diabetic rats and translates into a major alteration in cellular Mg^{2+} distribution; (2) this loss of Mg^{2+} impairs the ability of cardiac myocytes to extrude Mg^{2+} following β-adrenergic stimulation, and (3) insulin supplementation can restore Mg^{2+} homeostasis and transport in cardiac cells. The results reported here indicate that diabetes onset results in a time-dependent loss of Mg^{2+} that parallels a decrease in cellular ATP and protein content, and in the inability of cardiac cells to extrude Mg^{2+} following β-adrenergic stimulation. All these processes are restored following 2 weeks of insulin supplementation.

MATERIALS AND METHODS

Materials

Collagenase (CLS-I) was from Worthington (NJ). All other chemicals, including cell permeant cAMP, isoproterenol and epinephrine were of analytical grade (Sigma, St Louis).

Induction of Diabetes

Male Sprague-Dawley rats (200–220 g body weight) were rendered diabetic by a single

i.p. injection of streptozotocin (65 mg/kg body weight in citrate buffer, pH 4.0). The insurgence of diabetes was determined as the appearance of glucose in the urine, which occurred within 36 h from streptozotocin administration. Urinary level of glucose was measured by glucose strips (Fisher, Pittsburgh, PA) twice per week throughout the duration of our study to monitor diabetes progression. For the first 4 or 8 weeks of our study no insulin supplement was provided. After 8 weeks, the diabetic animals were divided into two groups, one of which received 2 U/50 g body weight/day Ultralent insulin to normalize glycemia. The animals were maintained on 12 h light/no light cycle and had free access to food and water. Less than 5% of the animals injected with streptozotocin did not become diabetic. These animals were separately analyzed to exclude that some of the observed effects were due to non-specific streptozotocin effects. Mortality was minimal, accounting for 1–2% of all the injected animals, and mostly occurred within the first week following streptozotocin administration.

Animals Ethics

Animals were maintained and handled in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council 1996), as approved by the Animal Resource Center at Case Western Reserve University, Cleveland, Ohio.

Determination of Cardiac Ion Content

At 2, 4, and 8 weeks following diabetes induction, diabetic and age-matched non-diabetic rats were anesthetized by intra-peritoneal injection of a saturated sodium pentobarbital solution (50 mg/kg body weight). The abdomen was opened and a volume of urine and blood (in a heparinized syringe) were collected from the bladder and the inferior vena cava, respectively. The heart was harvested, rinsed in 250 mM sucrose, blotted on absorbing paper, weighted and homogenized in 10% HNO_3. After overnight digestion, aliquots of the acid extracts were transferred in microfuge tubes, and the denaturated protein was sedimented at 8,000*g* for 5 min. The acid supernatants were removed and assessed for Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+} content by atomic absorbance spectrophotometry (AAS) in a Perkin-Elmer 3100 after proper dilution (Table I). Cation content was normalized for protein content, measured according to the procedure of Lowry et al. [1951], and for tissue weight.

Langendorff Perfusion

At the time points indicated above, diabetic and age-matched non-diabetic rats were anesthetized as previously described. The chest was opened and the heart rapidly excised at the aorta arch. The aorta was mounted on a truncated 16 gauge needle and the heart was flushed with a medium containing (mM): NaCl

TABLE I. Cation Content in Cardiac Tissue and Urine at Various Time Points After Diabetes Induction

	1 week		2 weeks		4 weeks	
	Non-Diab.	Diab.	Non-Diab.	Diab.	Non-Diab.	Diab
Heart						
Mg^{2+}	148.0 ± 4.4	132.4 ± 3.4	151.8 ± 2.1	121.6 ± 3.4^{a}	147.4 ± 3.2	$98.3 \pm 3.4^{a,b}$
K^{+}	1700.3 ± 40.8	1519.1 ± 39.8^{c}	1722.3 ± 46.2	$1412.4 \pm 39.8^{a,b}$	1711.3 ± 23.3	$1241.3 \pm 39.9^{a,b,d}$
Na^{+}	297.1 ± 2.3	342.7 ± 4.7^{a}	306.7 ± 7.6	$403.1 \pm 13.4^{a,b}$	301.9 ± 5.0	$415.2 \pm 9.4^{a,b}$
Ca^{2+}	25.9 ± 1.2	28.9 ± 2.9	25.9 ± 0.4	31.6 ± 0.5^{a}	25.9 ± 1.6	32.1 ± 1.7^{a}
Urine						
Mg^{2+}	5.2 ± 0.3	7.9 ± 0.4^{a}	5.2 ± 0.2	8.9 ± 0.5^{a}	5.4 ± 1.0	8.3 ± 0.4^{a}
K^{+}	71.8 ± 0.6	74.9 ± 0.5	71.2 ± 0.7	77.8 ± 0.8^{c}	72.8 ± 2.9	$89.7 \pm 4.5^{a,b}$
Na^{+}	148.3 ± 2.4	136.7 ± 4.5	147.1 ± 1.7	118.6 ± 3.1^{a}	150.6 ± 9.5	$98.8 \pm 6.7^{a,b}$
Ca^{2+}	6.6 ± 0.2	6.4 ± 0.2	6.6 ± 0.1	6.1 ± 0.09^{c}	6.4 ± 0.1	5.7 ± 0.09^{a}

Data are expressed as nmol/mg protein (heart) or mmol/dl (urine). Data are means + SE of six different animals for both non-diabetic (Non-Diab.) and diabetic (Diab.) experimental group at each time point.
N.A., not applicable.
[a]Statistical significant ($P < 0.001$) versus corresponding non-diabetic value.
[b]Statistical significant ($P < 0.001$) versus corresponding diabetic value at 1 and 2 weeks.
[c]Statistical significant ($P < 0.02$) versus corresponding non-diabetic value.
[d]Statistical significant ($P < 0.02$) versus corresponding diabetic value at 2 weeks.

120, KCl 3, $CaCl_2$ 1, $MgCl_2$ 0.8, K_2HPO_4 1.2, $NaHCO_3$ 12, glucose 15, Hepes 10, pH 7.2 at 37°C, equilibrated with an O_2:CO_2 (95:5, v/v) gas mixture (*Perfusion medium*). The heart was then connected to a perfusion system and retrograde perfused in a Langerdoff manner with the medium indicated above equilibrated with O_2:CO_2 (95:5, v/v) at a flow rate of 7 ml/g/min, at 37°C [Romani and Scarpa, 1990a]. After a few minutes of equilibration, the perfusion medium was switched to one having a similar composition but devoid of Mg^{2+} (*Mg^{2+}-free medium*). The contaminant Mg^{2+} present in the medium was measured by AAS and found to range between 5 and 7 μM. Samples of the perfusate were collected at 30 s intervals, and the Mg^{2+} content measured by AAS. The first 10 min provided a baseline for the subsequent addition of adrenergic agonist. Isoproterenol (10 μM), epinephrine (5 μM) or 8-Br-cAMP (250 μM) were dissolved directly into the perfusion medium, and administered for the time reported in the figures (see Fig. 1 for a schematic of the protocol). Pharmacological doses of the agonists were used to exclude reduced adrenoceptor responsiveness. To estimate the total amount of Mg^{2+} extruded from the organ, the Mg^{2+} content in the perfusate of the last six points prior to the adrenergic agonist addition was averaged and subtracted from each of the time points under the curve of efflux. The net amount of Mg^{2+} mobilized into the perfusate (nmol/ml) was then calculated taking into account the perfusion rate (7 ml/g /min) and the time of collection (30 s), and expressed as μmol. The residual Mg^{2+} content in perfused hearts was also calculated in tissue homogenate as described previously. The absence of cell damage was assessed by enzymatically measuring 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 according to published protocol [Romani and Scarpa, 1990a]. At the end of the perfusion procedure, the heart was removed from the perfusion system, blotted on absorbing paper, and weighed to normalize Mg^{2+} extrusion per gram of tissue. This procedure was necessary to take into account the reduction in heart weight observed in diabetic animals.

Cardiac Myocytes Isolation

Cardiac ventricular myocytes were isolated by collagenase digestion according to our published protocol [Romani and Scarpa, 1990a]. After isolation, myocytes were resuspended in the *perfusion medium* indicated previously, at the final concentration of 2×10^5 cells/ml containing 0.8 mM $MgCl_2$, and kept at room temperature, under constant flow of O_2:CO_2 95:5 until used. Cell viability, assessed by LDH release and maintenance of rod shape was found to be similar for diabetic and non-diabetic myocytes (i.e., $79 \pm 3\%$ and $78 \pm 4\%$ for non-diabetic and diabetic

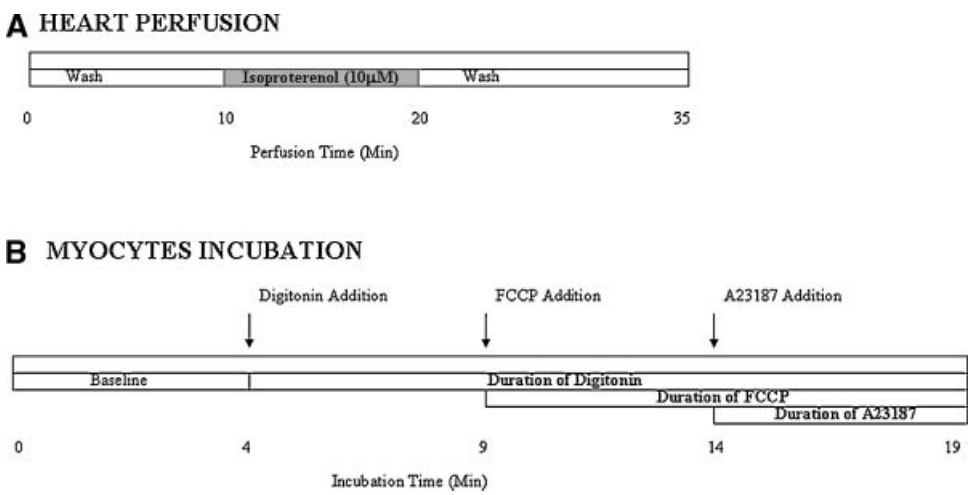

Fig. 1. Schematic of the perfusion and incubation protocols. The cartoon illustrates the schematic of agonist administration in the perfusion protocol (**A**) and the sequence of addition of digitonin, uncoupler and ionophore in the cell incubation system (**B**), as described under Materials and Methods Section.

cells, respectively, n = 9), and did not change significantly over the course of 4 h (78 ± 5% and 76 ± 6%, respectively, n = 8).

To determine Mg^{2+} transport, 1 ml of myocytes suspension was transferred to a microfuge tube, and the cells were rapidly sedimented at 600*g* for 30 s. The pellet was washed once with 1 ml of Mg^{2+}-free medium. After washing, the myocytes were transferred to 8 ml of Mg^{2+}-free incubation medium, pre-warmed at 37°C, and incubated under continuous O_2:CO_2 flow and stirring. After 2 min of equilibration, isoproterenol or cell permeant cAMP was added to the incubation system. At 2 min intervals, 0.7 ml of incubation mixture was withdrawn in duplicate, and the cells sedimented in microfuge tubes. The supernatants were removed and their Mg^{2+} content was determined by AAS. The cell pellets were digested overnight in 10% HNO_3. The denaturated protein content was sedimented at 8,000*g* for 5 min in microfuge tubes and the Mg^{2+} content of the acid extract was measured by AAS.

Cellular Mg^{2+} Distribution

To estimate the total cellular Mg^{2+} content and its distribution among cytoplasm, mitochondria and other cellular organelles (mainly but not only sarcoplasmic reticulum), cardiac myocytes isolated from diabetic and non-diabetic animals were sedimented, washed and incubated in Mg^{2+}-free medium as described previously. Digitonin (50 μg/ml final concentration), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 2 μg/ml), and A23187 (2 μg/ml) were sequentially added to the incubation system at 5 min interval (Fig. 1B), and aliquots of the incubation mixture withdrawn and sedimented at 10,000*g* for 2 min. The Mg^{2+} content of the supernatant was measured by AAS. Residual Mg^{2+} content in the cell pellets was measured by AAS after acid digestion performed as reported in the previous paragraph. The Mg^{2+} content present in the cell pellet or in the extracellular space prior to the addition of any stimulatory agent was calculated and subtracted from the following time points of incubation to determine the net amount of Mg^{2+} retained within the cell or released into the incubation medium.

Sarcolemmal Vesicles Purification and Loading

Sarcolemmal (SL) vesicles were isolated according to the procedure of Velema and Zaagsma [1981], as described in detail elsewhere [Cefaratti and Romani, 2007]. Magnesium loading was attained by diluting SL vesicles in 5 volumes of 250 mM sucrose, 20 mM HEPES/TRIS, pH 7.4 (*incubation medium*), in the presence of 20 or 10 mM $MgCl_2$. These concentrations are required to effectively determine Mg^{2+} transport in purified vesicles [Cefaratti et al., 1998]. The suspension was homogenized by 4 strokes in a Thomas C potter. Vesicles were sedimented at 20,000 rpm × 20 min in a Sorvall SS34 rotor at 4°C to remove excess, non-trapped cation. The pellet was resuspended in the incubation buffer described above at the final concentration of 5 mg protein/ml, and stored in ice until used.

To measure Mg^{2+} transport, Mg^{2+}-loaded SL vesicles were incubated at the final concentration of 250 μg protein/ml in the incubation medium described above. Following 3 min of equilibration, 0.7 ml of the incubation mixture were withdrawn in duplicate at time t = 0 and t = 2 min, and sedimented at 14,000 rpm for 1 min in microfuge tubes. Following withdrawal of t = 2 min sample, 20 mM NaCl or 500 μM $CaCl_2$ (concentrations that elicit maximal Mg^{2+} extrusion [Cefaratti and Romani, 2007]) were added to the extravesicular milieu to elicit Mg^{2+} extrusion, and the incubation continued for 6 min. Aliquots (0.7 ml) of the incubation mixture were withdrawn in duplicate at 1 min intervals (the earliest time we could reliably determine) and sedimented in microfuge tubes. The supernatants were removed and Mg^{2+} content measured by AAS. The SL pellets were digested overnight in 10% HNO_3, and their Mg^{2+} contents were also measured by AAS upon sedimentation of denaturated protein. For simplicity, Mg^{2+} transport in SL vesicles is reported as net change. The amount of Mg^{2+} extruded in the extravesicular space at times t = 0 and t = 2 min was calculated, averaged and subtracted from the following time points of incubation. The amount of Mg^{2+} retained in the pellet was calculated in a similar manner.

SL Purity

The purity and orientation of Mg^{2+}-loaded SL vesicles was determined by measuring Na^+/K^+-ATPase and 5′-nucleotidase activity, as described previously [Cefaratti et al., 1998; Cefaratti and Romani, 2007], in the absence or in the presence of Triton X-100. For both enzymes, the activity of vesicles incubated in

the absence of detergent was referred to the activity measured in vesicles treated with detergent (100%). Approximately 90% of SL vesicles were in the '*inside-in*' configuration following loading with Mg^{2+}. For simplicity, only the enzymatic activities in the presence of Triton-X 100 are reported in this study (Table II). The Na^+/K^+-ATPase activity was determined as the ouabain-sensitive fraction of the total ATPase activity [Cefaratti et al., 1998; Cefaratti and Romani, 2007]. Consistent with previous reports in the literature [Ng et al., 1993; Ver et al., 1997], the Na^+/K^+-ATPase activity was decreased by approximately 50% under diabetic conditions in both total homogenate and SL vesicles. Contamination by mitochondria or sarcoplasmic reticulum components was assessed via cytochrome *c*-oxidase and cytochrome *c*-reductase activities, respectively, and found to be negligible (*not shown*), consistent with the previous report by Velema and Zaagsma [1981].

Additional Procedures

Aliquots of the perfusate were collected at 1 min interval, and LDH activity measured by enzymatic kit (Sigma) sensitive to detect changes in the μU/ml range, and expressed as U/L. Similar determinations were carried out in isolated cardiac myocytes. In the latter case, LDH activity was also assessed as a percentage of the total amount of the enzyme releasable from digitonin-permeabilized cells.

Cellular ATP content level was measured in isolated myocytes by luciferin-luciferase assay as reported previously [Tessman and Romani, 1998]. Cyclic AMP level was determined in aliquots of cell extract by ^{125}I-RIA (Amersham) as reported previously [Tessman and Romani, 1998].

Statistical Analysis

The data are reported as mean ± 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 at least $P < 0.05$.

RESULTS

Cation Content

Two weeks after streptozotocin injection, diabetic rats exhibited a ≥15% decrease in body weight as compared to age-matched non-diabetic animals (~232 ± 8 g vs. 265 ± 7 g, respectively, n = 6). The decrease in body weight in diabetic rats progressed with time, reaching >30% at 4 weeks (~275 ± 11 vs. 392 ± 9 g in diabetic and non-diabetic, respectively, n = 8 for both groups, $P < 0.05$) and ~45% at 8 weeks from diabetes induction. Four-week diabetic animals displayed a fourfold increase in glycemia as compared to age-matched controls (378 ± 28 vs. 92 ± 8 mg/100 ml, respectively, n = 8, $P < 0.001$). Consistent with previous reports [Wallach and Verch, 1987; Resnick et al., 1993], total cardiac cation content markedly changed following diabetes onset. As shown in Table I, both total Mg^{2+} and K^+ contents decreased by ~10% at 1 week whereas Na^+ and Ca^{2+} contents increased. The increase was more evident for Na^+ than for Ca^{2+}. The latter cation, in fact, showed a percent change that did not reach statistical significance at this time point. At 2 weeks following streptozotocin-injection, both Mg^{2+} and K^+ content

TABLE II. Enzymatic Activity in Sarcolemmal Vesicles From 4 Weeks Diabetic and Age-Matched Non-Diabetic Animals

	5′-Nucleotidase enrichment		Na^+/K^+-ATPase enrichment	
Total homogenate				
Non-diabetic	0.24 ± 0.02	N.A.	0.14 + 0.010	N.A.
Diabetic	0.21 ± 0.09	N.A.	0.06 ± 0.005[a]	N.A.
Sarcolemmal vesicles				
Non-diabetic	1.07 ± .0.50	4.5	0.75 ± 0.035	5.4
Diabetic	0.98 ± 0.046	4.5	0.38 ± 0.027[a]	6.3

The activities of 5′-nucleotidase and Na^+/K^+-ATPase are expressed as μmol Pi/mg protein/min. Na^+/K^+-ATPase activity was determined as the ouabain-sensitive fraction of the total ATPase activity [Cefaratti et al., 1998]. All experimental protocols were performed in the presence of 1% TritonX-100 for total activity as previously described [Cefaratti et al., 1998]. Sarcolemmal vesicles enrichment in enzyme activity is calculated versus the corresponding value in the respective homogenate. All data are reported as mean ± SE; n = 5 for all conditions.
Contamination of sarcolemmal vesicles preparation by sarcoplasmic reticulum or mitochondria was negligible, and values are not reported for simplicity.
N.A., not applicable.
[a]Statistical significant ($P < 0.001$) versus corresponding non-diabetic value.

within cardiac tissue were decreased by ~20% compared to age-matched non-diabetic rats while Na^+ content had increased proportionally (Table I). Total Ca^{2+} content was also significantly increased. The decrease in Mg^{2+} and K^+ content reached approximately 30% in animals diabetic for 4 weeks. At all the indicated time points, tissue Mg^{2+} and K^+ content were similarly lower when compared to the levels determined in tissues of weight-matched non-diabetic rats (*not shown*), thus excluding that the observed changes reflected differences in body development. Serum Mg^{2+} content in diabetic animals showed a downward trend compared to age-matched animals at both 4 and 8 weeks but the difference was not statistically significant (*not shown*). In contrast, the urine content of Mg^{2+} and K^+ increased by ~70% and ~25%, respectively, by week 4 after diabetes onset (Table I). Less than 5% of all streptozotocin-injected animals did not develop hyperglycemia and glucosuria, and presented cardiac cation content similar to that of non-diabetic animals. The values from these animals are not reported here for simplicity (*not shown*).

The decrease in whole tissue Mg^{2+} content was confirmed at the level of collagenase-dispersed cardiac ventricular myocytes. As Figure 2A shows, cardiac myocytes isolated at different time points following diabetes induction presented a progressive decrease in total cellular Mg^{2+} content (i.e., minus 46% at 4 weeks after streptozotocin injection) as compared to cells isolated from age-matched non-diabetic animals. The decrease in Mg^{2+} content continued, though at slightly slower pace between 4 and 8 weeks (~50% at 8 weeks) from diabetes induction (*not shown*), suggesting that the cells attained a new steady state level by 4 weeks, not changing significantly at the latest time point of our study. For this reason, the majority of the experiments described here were performed on 4-week diabetic animals. Determination of cellular Mg^{2+} distribution within the cell was conducted by using pharmacological agents added sequentially that accessed Mg^{2+} contained within the cytoplasm (digitonin), mitochondria (FCCP as mitochondria uncoupler) and in the remaining cellular pools including sarcoplasmic reticulum and nucleus (A23187 ionophore). The use of FCCP to mobilize Mg^{2+} from the mitochondria finds its rationale in the observation that inhibition of mitochondrial respiration by this uncoupler results in a marked Mg^{2+} efflux in rat heart mitochondria [Akerman, 1981]. We have previously used these pharmacological agents to estimate Mg^{2+} content within the mentioned pools in liver cells [Fagan et al., 2004] with results comparable to those obtained with more sophisticated techniques (e.g., EPXMA). The results, reported in Figure 2B, indicate that Mg^{2+} is lost from all cellular pools at week 1 following diabetes induction, the loss in mitochondria and extra-mitochondria pool(s) being more evident at later time points. Total protein (Fig. 3A) and ATP content (Fig. 3B) also declined with diabetes progression, an indication that cellular protein and ATP represent two of the main moieties forming a complex with Mg^{2+} within the cardiac myocyte. While ATP content did not decrease significantly over time, protein content continued to decline with diabetes progression.

Hormonal Mobilization of Cellular Mg^{2+}

The administration of catecholamine [Vormann and Gunther, 1987; Romani and Scarpa, 1990a] or isoproterenol [Romani et al., 1993] to perfused hearts or isolated cardiac myocytes results in a major mobilization of Mg^{2+} from cardiac cells into the extracellular space. Hence, diabetic hearts were perfused with isoproterenol to determine whether the observed decreased in cardiac Mg^{2+} content resulted in a defective Mg^{2+} mobilization by adrenergic agonists. As Figure 4A shows, β-adrenergic receptor mediated Mg^{2+} extrusion was significantly reduced in 4-week diabetic hearts. An estimate of the area under the curve of extrusion (calculated as indicated under Materials and Methods Section) indicates that the net amount of Mg^{2+} extruded from diabetic hearts decreased by ~68% at 2 weeks and by ~70% at 4-week after diabetes induction (Fig. 4B). A similar decrease in the total amount of Mg^{2+} extruded was also observed in hearts stimulated by epinephrine (5 μM, Fig. 4B). Isoproterenol and epinephrine-induced Mg^{2+} extrusion decreased ~75% in hearts from animal diabetic for 8 weeks (*not shown*). These results are not surprising as experimental evidence indicates that sarcolemmal distribution of Gαs and Gαi in diabetic cardiac myocytes changes significantly, affecting β-adrenergic receptor responsiveness and cAMP production [Roth et al., 1995]. To determine whether the decrease in Mg^{2+} extrusion from diabetic cardiac cells depended on a

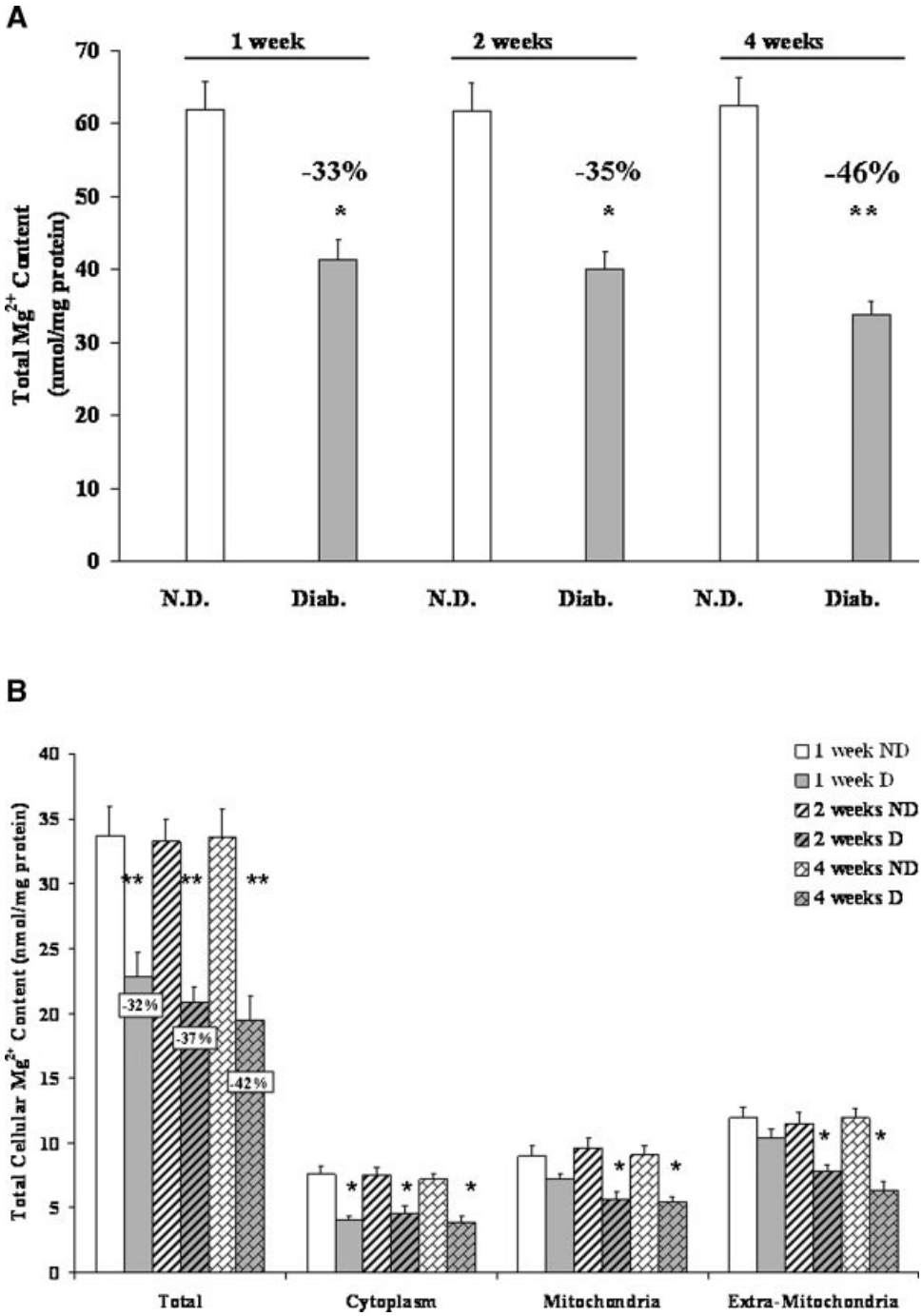

Fig. 2. Total Mg^{2+} content (**A**) and cellular Mg^{2+} distribution (**B**) in cardiac ventricular myocytes from diabetic animals. Cardiac ventricular myocytes were isolated by collagenase digestion from animals diabetic for 1, 2, and 4 weeks following streptozotocin-injection. Total cellular Mg^{2+} content (A) was determined by AAS and normalized per mg of protein as reported under Materials and Methods Section. The amount of Mg^{2+} present in the different cellular compartments (B) was estimated by sequentially treating collagenase-dispersed myocytes with digitonin (cytoplasm), mitochondrial uncoupler FCCP (mitochondria) and ionophore A23187 (post-mitochondrial) as described under Materials and Methods Section, and illustrated in Figure 1B. Data are means $\pm$ SE of six different preparations, each assessed in duplicate. **Statistical significant versus corresponding non-diabetic value at $P < 0.001$; *Statistical significant versus corresponding non-diabetic value at $P < 0.01$.

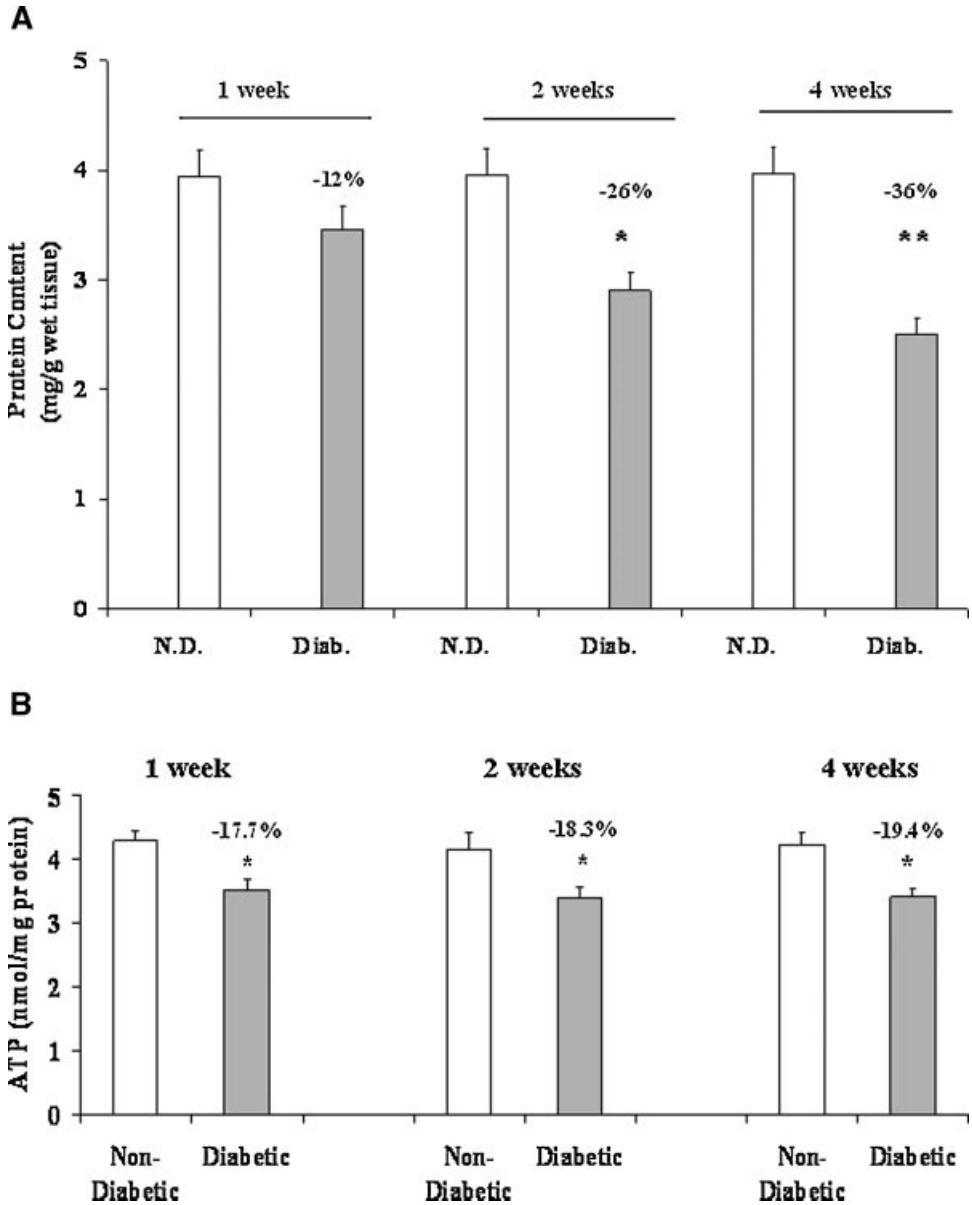

Fig. 3. Protein (**A**) and ATP content (**B**) in cardiac ventricular myocytes from diabetic animals. Collagenase-dispersed cardiac ventricular myocytes were isolated from animals diabetic for 1, 2, and 4 weeks diabetic following streptozotocin-injection. Total protein content (A) was determined by Lowry et al. [1951]. ATP content (B) was measured by luciferin-luciferase assay and confirmed by HPLC [Tessman and Romani, 1998]. Data are means ± SE of nine and six different preparations for protein and ATP content, respectively, each assessed in triplicate. **Statistical significant versus corresponding non-diabetic value at $P < 0.001$; *Statistical significant versus corresponding non-diabetic value at $P < 0.01$.

defective G-protein signaling or on the observed decrease in Mg^{2+} content, perfused hearts from diabetic animals were stimulated by 250 μM 8-Cl-cAMP, a dose reported to elicit Mg^{2+} extrusion from hearts [Romani et al., 2000] or livers [Fagan and Romani, 2000] under physiological conditions. As expected, the administration of this agent elicited a Mg^{2+} extrusion in non-diabetic hearts that was qualitatively (Fig. 5A) and quantitatively (Fig. 5B) similar to that elicited by isoproterenol under similar experimental conditions. In contrast, the profile of Mg^{2+} extrusion was significantly reduced in 4 weeks diabetic hearts (Fig. 5A). The estimate of the total net amount of Mg^{2+} extruded by cAMP resulted in a value that was 50–60% smaller than that observed in age-matched non-diabetic hearts (Fig. 5B). Net Mg^{2+} extrusion

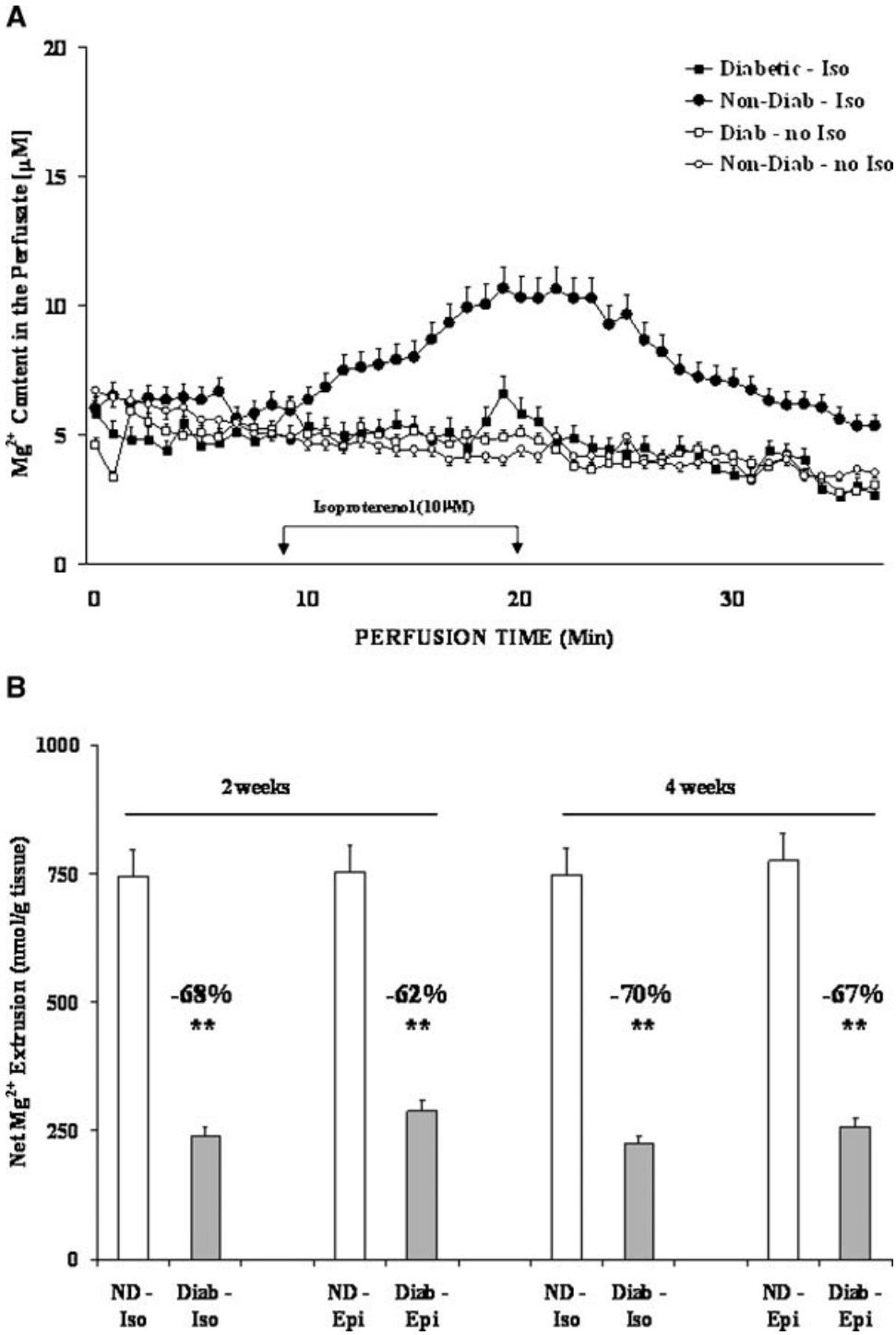

Fig. 4. Mg^{2+} extrusion from perfused hearts stimulated by β-adrenergic agonist. Hearts from 2- and 4-week diabetic animals and age-matched non-diabetic rats were perfused as described under Materials and Methods Section. At the time indicated in the figure, 10 μM isoproterenol (ISO, **A**), or 5 μM epinephrine (EPI—not shown) were directly dissolved into the perfusion medium and administered for 10 min. A: Reports the extrusion profile of isoproterenol-stimulated diabetic and non-diabetic hearts (4 weeks). The net amount of Mg^{2+} extruded from diabetic and non-diabetic hearts stimulated by 10 μM ISO or 5 μM EPI, calculated as described under Materials and Methods Section is reported in (**B**). The data are means ± SE of six different preparations for each experimental condition. A: All the data points relative to isoproterenol-induced Mg^{2+} extrusion in non-diabetic hearts are statistically significant versus the corresponding points of un-stimulated non-diabetic hearts and isoproterenol-stimulated diabetic hearts. Labeling is omitted for simplicity. B: **Statistically significant versus corresponding values of non-diabetic samples at $P < 0.001$.

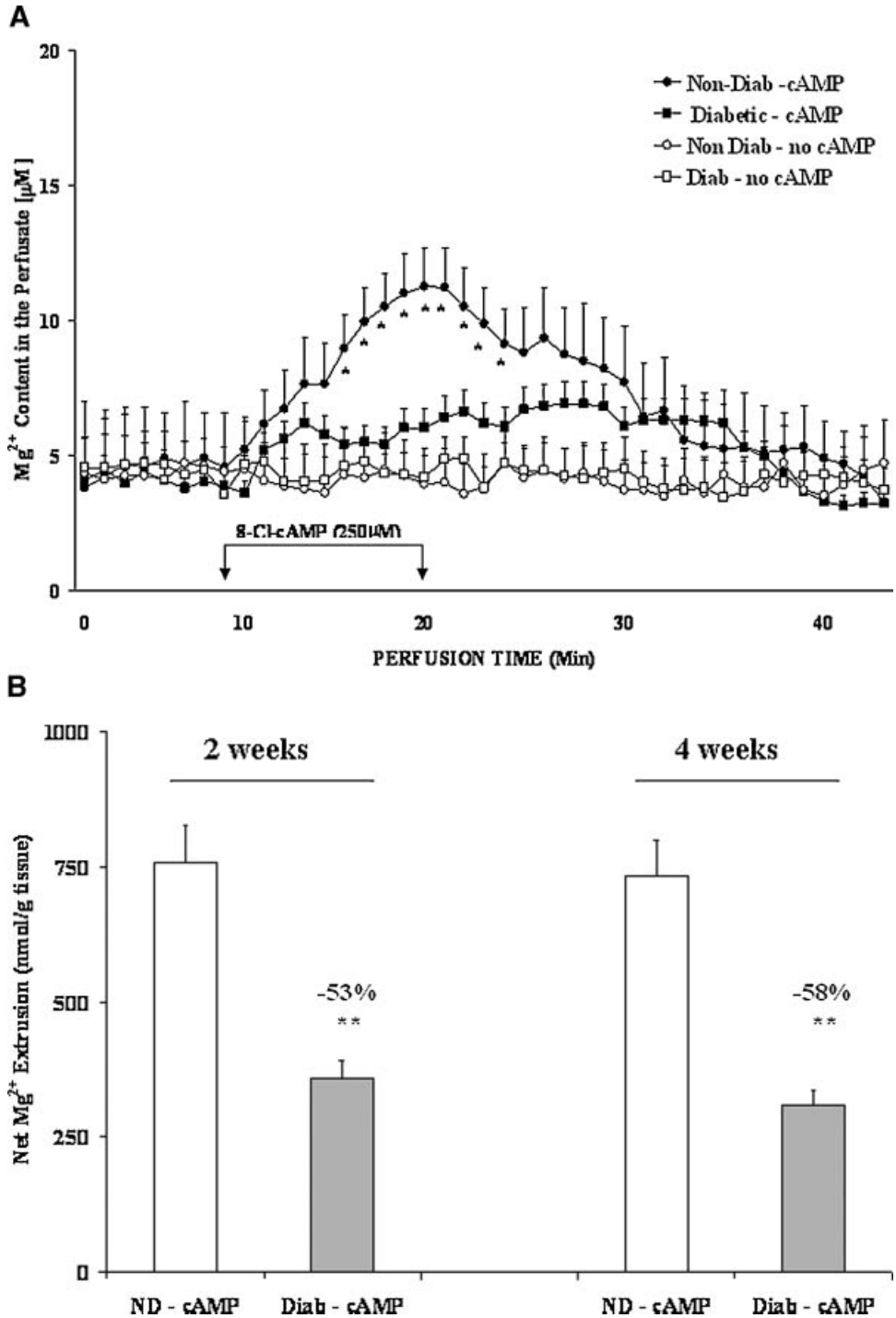

Fig. 5. Mg^{2+} extrusion from perfused hearts stimulated by 250 μM 8-Cl-cAMP. Hearts from 4 weeks diabetic animals and age-matched non-diabetic rats were perfused as described under Materials and Methods Section. After a few minutes of equilibration, 250 μM 8-Chloro-phenyl-thio-cyclic AMP (8-Cl-cAMP) was directly dissolved into the perfusion medium and administered for 10 min. The extrusion of 8-Cl-cAMP-stimulated diabetic and non-diabetic hearts (4 weeks) is reported in (**A**). The net amount of Mg^{2+} extruded from diabetic and non-diabetic hearts is reported in (**B**). The data are means ± SE of five different preparations for each experimental condition. A: All the data points relative to cAMP-induced Mg^{2+} extrusion in non-diabetic hearts are statistically significant versus the corresponding points of unstimulated non-diabetic hearts; *Statistically significant versus corresponding values of cAMP-stimulated diabetic hearts. B: **Statistically significant versus corresponding values of non-diabetic samples at $P < 0.001$.

decreased slightly more than 60% in perfused hearts from 8-week diabetic animals (*not shown*). It has to be noted that hearts from diabetic and non-diabetic animals were maintained in perfusion for a longer period of time (i.e., 45 min instead of 35 min as reported in Fig. 4A) due to the longer time necessary for Mg^{2+} extrusion to return to baseline following the administration of cell permeant 8-Cl-cAMP.

To exclude the possibility that the observed results depended on the cell heterogeneity of the whole heart, stimulatory experiments with isoproterenol, epinephrine or cAMP were performed in collagenase-dispersed cardiac ventricular myocytes, with comparable results (*not shown*). In dispersed cardiac myocytes, RIA determination of cAMP produced by isoproterenol stimulation indicated that cAMP level increased twofold in cardiac myocytes from non-diabetic animals (3.49 ± 0.09 and 8.04 ± 0.11 pmol/mg protein/5 min in unstimulated and stimulated myocytes, respectively, $n = 5$ for both experimental conditions, $P < 0.01$) but only ~65% in cells from 4 weeks diabetic animals ($3.43 + 0.07$ and $5.63 + 0.08$ pmol/mg protein/5 min in unstimulated and stimulated myocytes, respectively, $n = 5$ for both experimental conditions, $P < 0.01$ vs. both unstimulated myocytes and stimulated non-diabetic cardiac cells).

We have recently characterized the operation of Mg^{2+} transport mechanisms in purified cardiac SL vesicles [Cefaratti and Romani, 2007]. Hence, diabetic cardiac SL vesicles were purified to investigate whether the decrease in Mg^{2+} extrusion could be ascribed, at least in part, to the defective operation of the Na^+/Mg^{2+} exchange mechanism. The results reported in Figure 6A indicate that Mg^{2+}-loaded SL vesicles purified from 4-week diabetic animals extruded Mg^{2+} to an extent comparable to that observed in SL vesicles isolated from age-matched non-diabetic animals. Comparable results were obtained in vesicles isolated from 8-week diabetic animals (*not shown*). When SL vesicles from 4-week diabetic animals were loaded with 10 mM Mg^{2+}, a concentration resembling the 50% decrease in total cellular Mg^{2+} content observed in diabetic cardiac cells, the amplitude of Mg^{2+} extrusion, relative to vesicles loaded with 20 mM Mg^{2+}, was decreased by ~50% in both diabetic and non-diabetic SL (Fig. 6B).

Insulin Supplementation

Ultralente insulin (2U/50 g b.w.) was administered daily for 2 weeks to animals diabetic for 4 or 8 weeks. The treatment returned body and heart weight in both 4-week and 8-week diabetic animals to values similar to those observed in age-matched non-diabetic animals (e.g., 359.0 ± 5.6 vs. 383.7 ± 8.1 g b.w., and 1.2 ± 0.3 vs. 1.31 ± 0.5 g heart weight in insulin-treated 4-week diabetic insulin-treated and age-matched non-diabetic, respectively, $n = 5$ for each group). Similar percent difference (5–7%) was observed in 8-week diabetic animals versus age-matched non-diabetic animals. Two weeks of insulin treatment in 4-week (Fig. 7A) or 8-week (*not shown*) diabetic animals also returned cardiac Mg^{2+} content to the level observed in non-diabetic animals both in terms of total amount (Fig. 7A) and cellular distribution (Fig. 7B). Cellular protein content and ATP levels were completely restored (*not shown*). The return of Mg^{2+} content to normal level was further confirmed by the ability of isoproterenol, epinephrine or cAMP (Fig. 8) to mobilize a net amount of Mg^{2+} comparable to that mobilized in age-matched non-diabetic animals. Two weeks were the minimum period of time required for insulin treatment to restore tissue ATP, protein and Mg^{2+} content in diabetic animals.

As mentioned under Materials and Methods Section, streptozotocin-injected animals that did not develop diabetes were also analyzed for protein, ATP and Mg^{2+} content, as well as Mg^{2+} distribution and efflux. Cardiac cells from these animals contained levels of Mg^{2+}, protein and ATP comparable to those of pare-aged, non-diabetic (i.e., non-injected rats) and responded to isoproterenol administration by extruding an amount of Mg^{2+} similar to that mobilized from cardiac cells of non-diabetic animals (*not shown*). Taken together, these results exclude that the changes in Mg^{2+} homeostasis and transport observed in diabetic hearts depended on non-specific streptozotocin effects. As the values obtained in these animals were super-imposable to those observed in non-diabetic animals they are not reported here for simplicity.

DISCUSSION

Experimental and clinical evidence indicates that both type-I and type-II diabetes are characterized by a loss in tissue Mg^{2+} content [Wallach and Verch, 1987; Resnick et al., 1993].

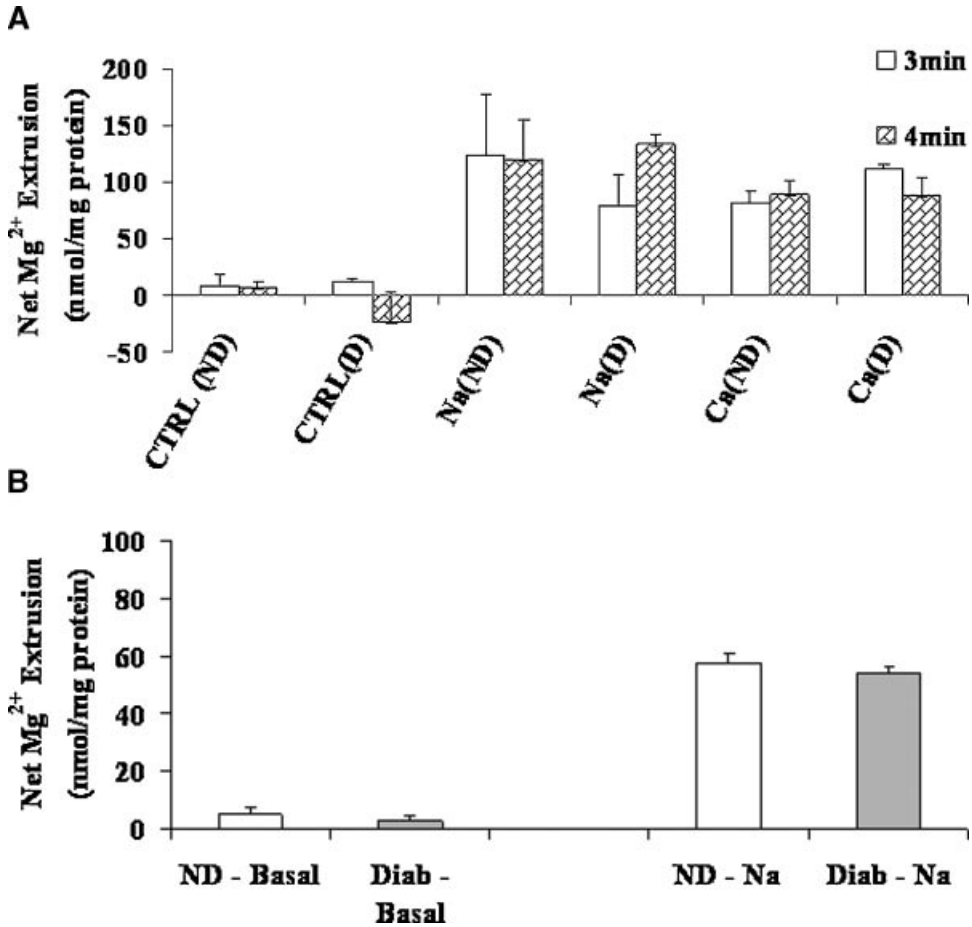

Fig. 6. Mg^{2+} transport in purified sarcolemmal vesicles. SL vesicles from 4 weeks diabetic and non-diabetic age matched animals were purified and loaded with 20 mM (**A**) or 10 mM Mg^{2+} (**B**) as indicated under Materials and Methods Section. Mg^{2+} extrusion was induced by addition of 20 mM Na^+ or 500 μM Ca^{2+} to the extravesicular milieu. For simplicity, only the extrusion at t = 4 min in the presence of 20 mM Na^+ is reported for vesicles loaded with 10 mM Mg^{2+} (B). Data are means ± SE of four different preparations for both diabetic and non-diabetic experimental groups, each performed in duplicate. A,B: All the data points relative to Na- and Ca^{2+}-induced Mg^{2+} extrusion in SL vesicles from non-diabetic and diabetic hearts are statistically significant versus the corresponding control points (i.e., unstimulated SL vesicles). Labeling is omitted for simplicity. Values of diabetic SL vesicles are not statistically significant as compared to the corresponding values in non-diabetic SL vesicles.

A decreased phosphorylation of insulin receptor and down-stream signaling molecules [Suarez et al., 1995] have been observed in Mg^{2+} depleted tissues. At the same time, a functional association between insulin receptor and the putative Na^+/Mg^{2+} exchanger has been reported [Ferreira et al., 2004]. Our laboratory has investigated changes in hepatic Mg^{2+} homeostasis from diabetic animals [Fagan et al., 2004], and provided experimental evidence for a defective accumulation within the hepatocyte. Aside from these studies, no information is available about the modality by which Mg^{2+} loss occurs in diabetic tissues including hearts, either from animals or patients, and the short- and long-term implications of Mg^{2+} deficiency for tissue metabolic processes under diabetic condition.

The present study was undertaken to determine how cardiac Mg^{2+} homeostasis and transport is altered under diabetic conditions, and generate the necessary background for future mechanistic studies aimed at elucidating the implication(s) of a decrease in Mg^{2+} content for cardiac metabolism and function.

Cardiac Mg^{2+} Homeostasis

The results reported here indicate that the onset of type-I diabetes results in a time-dependent loss of Mg^{2+} from cardiac tissue. The Mg^{2+} loss is associated with a comparable decrease in tissue K^+, protein and ATP content.

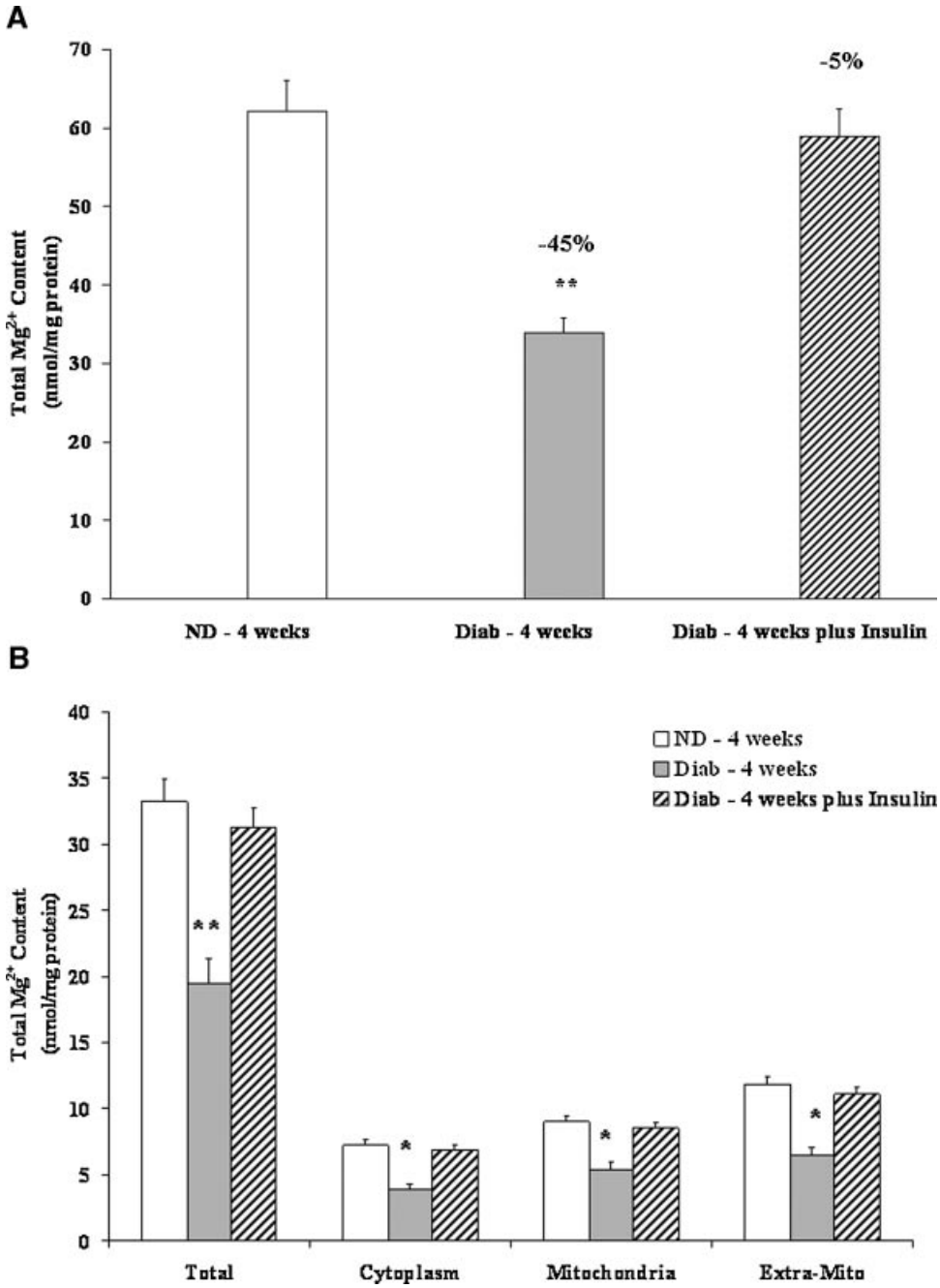

Fig. 7. Total Mg^{2+} content (**A**) and cellular Mg^{2+} distribution (**B**) in cardiac ventricular myocytes from insulin-treated diabetic animals. Cardiac ventricular myocytes were isolated by collagenase digestion from animals diabetic for 4 weeks diabetic following streptozotocin-injection and treated with daily injection of insulin for 2 weeks. Total cellular Mg^{2+} content (A) was determined by AAS and normalized per mg of protein as reported under Materials and Methods Section. The amount of Mg^{2+} present in the different cellular compartments (B) was estimated by sequentially treating collagenase-dispersed myocytes with digitonin, FCCP and A23187 as indicated in the legend to Figure 2. Data are means ± SE of five different preparations, each assessed in duplicate. A: **Statistical significant versus corresponding values in non-diabetic and insulin-treated diabetic samples, at $P < 0.001$. B: **Statistical significant versus corresponding non-diabetic value at $P < 0.001$; *Statistical significant versus corresponding non-diabetic value at $P < 0.01$.

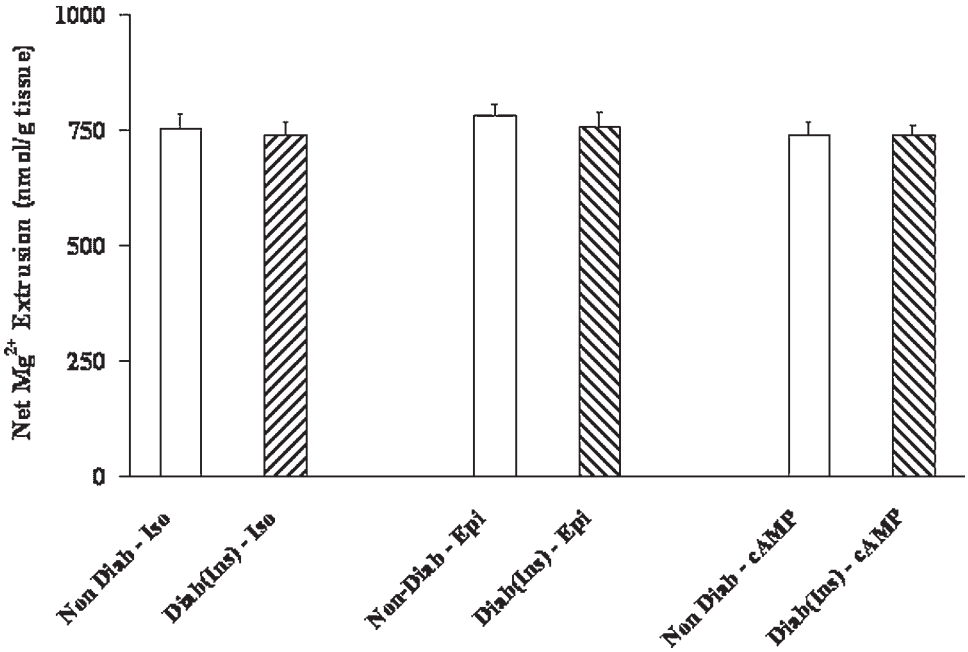

Fig. 8. Net Mg^{2+} extrusion from perfused hearts stimulated by β-adrenergic agonist. Hearts from 4 weeks diabetic animals treated with insulin for 2 weeks and age-matched non-diabetic rats were perfused as described under Materials and Methods Section with 10 μM ISO, 5 μM EPI or 250 μM 8-Cl-cAMP (cAMP). The net amount of Mg^{2+} extruded from the perfused hearts was calculated as described under Materials and Methods Section. The data are means ± SE of four different preparations for each experimental condition. All the reported values for non-diabetic animals and diabetic animals receiving insulin treatment are statistically significant versus corresponding values in diabetic animals as reported in Figure 3. Labeling is omitted for simplicity.

The decrease in ATP, protein, Mg^{2+} and K^+ contents are unlikely to depend on cell swelling and gain in water content for two reasons: (1) the hearts of type-I diabetic animals are usually smaller in size and weight (~25% and ~28% loss at 2 and 4 weeks, respectively [Lashin et al., 2006]), and (2) these decreases remain essentially unmodified when normalized per g of tissue rather than per mg of protein (*not shown*). The increase in Na^+ and possibly Ca^{2+} content further excludes the possibility that the observed decrease in Mg^{2+}, K^+, ATP and protein level depends on cardiac atrophy observed in diabetic animals, and is consistent with published results [Wallach and Verch, 1987; Resnick et al., 1993; Fagan et al., 2004]. The changes in cardiac cation content are significant when compared to those observed in both age-matched and weight-matched non-diabetic animals. The increased elimination of Mg^{2+} with the urine in diabetic animals can be explained by the fact that the ascending limb of Henle's loop, which reabsorbs more than 95% of plasma Mg^{2+} under physiological conditions [Quamme, 1993], is the site where various hormones, including insulin, act to enhance Mg^{2+} reabsorption [Quamme, 1993]. In the absence of insulin, the ability of this portion of the nephron to reabsorb Mg^{2+} would be largely compromised or completely lost, resulting in an increased elimination of Mg^{2+} in the urine. Due to the polyuria present in diabetic animals, the amount of Mg^{2+} and K^+ eliminated with the urine can easily account for the loss of these elements from the heart as well as other tissues. We did not observe, however, a decline in plasma Mg^{2+} level as reported by other laboratories [Wallach and Verch, 1987; Resnick et al., 1993]. This difference may depend on the time elapsed from diabetes onset, the age of the animals or the basal Mg^{2+} level at the time of diabetes induction. More specific studies need to be carried out to elucidate this point.

Mg^{2+} Homeostasis Within the Cardiac Myocytes

The results obtained in perfused hearts or isolated myocytes provide some insight about the cellular modification of Mg^{2+} homeostasis that ultimately results in a loss of Mg^{2+} content within the tissue. First, the loss in Mg^{2+} content detected in isolated cells appears to be larger than that measured in total heart extract. This

discrepancy may reflect the cell heterogeneity of the whole heart and the fact that although Mg^{2+} is lost in cardiac myocytes, the cation is still retained, at least in part, in the interstitial space within the whole organ. Second, the loss of Mg^{2+} appears to affect all organelles and compartments, including cytoplasm (Fig. 2B). The depletion in cytosolic Mg^{2+} (~35%, Fig. 2) is quantitatively similar to the combined loss in total protein (25–35% Fig. 3A) and ATP (15–20%, Fig. 3B) content, suggesting that Mg^{2+} in the cytoplasm and within compartments is mainly regulated by binding to proteins and adenine phosphonucleotides [Scarpa and Brinley, 1981]. Based on a total ATP content of 4 mM, a 15% decrease in ATP corresponds to ~600 μM Mg^{2+} dissociating from the complex. The degradation of ATP to ADP and AMP results in a marked decrease in the association constant of these moieties to Mg^{2+} (i.e., from ~80 μM for MgATP to ~8.13 mM to MgAMP). This implies that as ATP degrades a significant amount of Mg^{2+} becomes unbound and is ultimately extruded from the myocyte. A similar pattern has been observed in diabetic hepatocytes [Fagan et al., 2004]. As already speculated in the case of the liver, cardiac cells must possess a specific albeit still unidentified mechanism that senses the increase in $[Mg^{2+}]_i$, and determines the extrusion of excess Mg^{2+} across the cell membrane. The transient increase in cytosolic Mg^{2+} content as a result of ATP degradation may also have inhibitory effects on Mg^{2+} entry mechanisms such as the TRPM7 channel sensitive to changes in Mg^{2+} concentration [Gwanyanya et al., 2004].

The changes in Mg^{2+} content within the cellular compartments, on the other hand, can represent a secondary adjustment to the changes in cytosolic bound/free Mg^{2+}, or be the result of an impaired organelle functioning under diabetic conditions. It is well documented that Mg^{2+} regulates directly or indirectly numerous enzymes in the cytoplasm (e.g., adenylyl cyclase [Pignatti et al., 1993]) and within organelles, including mitochondrial dehydrogenases [Panov and Scarpa 1996] and reticular ATPases and Ca^{2+} release [Duggleby et al., 1999]. In addition, Howarth et al. [1994] have reported that a reduced Mg^{2+} extrusion from cardiac cells following adrenergic stimulation significantly hampers myocardial contractility. Hence, it can reasonably be speculated that a loss of Mg^{2+} from the cytoplasm and within organelles can contribute to some of the cardiac contractile and metabolic changes observed under diabetic conditions.

Lack of Insulin and Mg^{2+} Homeostasis

The lack of insulin occurring under type-I diabetes can decrease cellular (cardiac) Mg^{2+} content in several ways. First, insulin promotes cellular K^+ uptake, which is essential for cellular Mg^{2+} accumulation to occur [Dai et al., 1997]. The observed reduction in K^+ content (Table I), associated to a decreased Na^+/K^+-ATPase function (Table II), will increase cellular Na^+ content and have a major repercussion on cellular Mg^{2+} content. Second, insulin receptor and Na^+/Mg^{2+} exchanger operation appear to be tightly coupled [Suarez et al., 1995]. Thus, a decrease in circulating insulin level will alter the operation of the exchanger, contributing to the decrease in cellular Mg^{2+} content and the increase in Na^+ level. Third, in the absence of insulin, proteolysis is no longer inhibited, resulting in a decrease in protein content and neosynthesis, and consequently in Mg^{2+} level within cardiac cells. An interesting corollary of the latter observation is that under physiological conditions Mg^{2+} content is maintained within the cell at a concentration optimal for protein synthesis [Terasaki and Rubin, 1985]. In this regard, an adequate level of cellular Mg^{2+} appears to be required for the stabilization of tRNA [Kalpaxis and Drainas, 1992; Oliva et al., 2007], 70S ribosomal complex [Hosaka et al., 2004], and mRNA [Lambert and Draper, 2007], at least in certain cell types. Therefore, a decrease in cellular Mg^{2+} content, especially in cytoplasm and sarcoplamic reticulum can further impair protein synthesis and homeostasis, resulting in a vicious cycle that ultimately affects both protein and Mg^{2+} homeostasis within the myocyte.

Altered Mg^{2+} Extrusion

One of the direct consequences of the decrease in cellular Mg^{2+} content is that the administration of β-adrenergic receptor agonists (isoproterenol or catecholamine) elicits a reduced mobilization of Mg^{2+} from the perfused heart or the isolated cells. Experimental evidence suggests that the primary mechanism for Mg^{2+} extrusion in cardiac cells is the putative Na^+/Mg^{2+} exchanger previously described [Flatman, 1991], activated via cAMP-mediated phosphorylation [Gunther and Vormann, 1992]. The

expression of β-adrenergic receptor [Altan et al., 2007] and the generation of cAMP [Roth et al., 1995] are both compromised under diabetic conditions. In particular, cellular cAMP formation is decreased as a result of an altered Gαs and Gαi distribution in the sarcolemma [Roth et al., 1995]. Hence, it is not surprising that Mg^{2+} extrusion from diabetic cardiac cells is also significantly hampered. The results obtained in hearts perfused with 8-Cl-cAMP and purified SL vesicles further suggest that the decrease in cellular Mg^{2+} content, which affects all the compartments to a similar extent, contributes to the decrease in β-adrenergic receptor mediated Mg^{2+} extrusion observed in diabetic cardiac cells, even when cAMP level is not a limiting factor. The extrusion of Mg^{2+} is normal in diabetic SL vesicles loaded with 20 mM Mg^{2+}, that is, a 'physiological' Mg^{2+} level (Fig. 6A) while it is decreased in diabetic hearts even when perfused with 8-Cl-cAMP (Fig. 5). This observation suggests that within the myocyte cAMP has the double role of activating the Mg^{2+} extrusion mechanism and favoring Mg^{2+} mobilization from intra-cellular pool(s), the latter aspect being more severely affected under diabetic conditions. This possibility would be consistent, to some extent, with the recent evidence of a specific cAMP compartmentation within cardiac myocytes [Iancu et al., 2007]. Further studies are required to elucidate how exactly Mg^{2+} mobilization occurs, and which organelle(s) it eventually targets. It could be argued that loading sarcolemmal vesicles with 20 mM Mg^{2+} does not fully mimic Mg^{2+} content within cardiac cells, in that Mg^{2+} is compartmentalized and bound to ATP or protein within the cell. It has to be noted, however, that Mg^{2+} extrusion occurs to a comparable extent both in the absence and in the presence of intravesicular entrapped ATP [Cefaratti et al., 1998], thus indicating that binding to adenine phosphonucleotides is not a rate limiting factor for Mg^{2+} extrusion. In addition, the observation that Mg^{2+} extrusion is reduced in diabetic and non-diabetic SL vesicles loaded with 10 mM Mg^{2+}, a condition resembling the reduced Mg^{2+} content within diabetic myocytes, suggest that the transport rate across the Na^+/Mg^{2+} exchanger is a direct function of the amount of Mg^{2+} present within the vesicle (and the cell). As the amplitude of Mg^{2+} extrusion is similar in diabetic and non-diabetic SL vesicles, it can reasonably be concluded that the reduced Mg^{2+} extrusion observed in cardiac cells from diabetic animals is not the result of a loss in the number of putative Mg^{2+} transporters.

Taken together, these results indicate that under diabetic conditions cellular Mg^{2+} homeostasis is altered within the myocyte as a result of the decreased buffering capacity by cellular proteins and ATP. In turn, the decrease in Mg^{2+} content affects the amount of Mg^{2+} that can be extruded from the cell via the Na^+/Mg^{2+} exchanger. The trend towards an increase in total cellular Ca^{2+} level and/or the altered Ca^{2+} cycling between cytoplasm and sarcoplasmic reticulum could play an additional role in that Ca^{2+} would now compete with Mg^{2+} for the same protein binding site, as proposed by Koss et al. [1993].

Irrespective of the mechanisms involved, the altered cellular Mg^{2+} content and distribution as well as the response to β-adrenergic agonist or cell permeant cAMP are restored following 2 weeks of insulin supplementation. Insulin administration for less than 2 weeks did not fully restore Mg^{2+} homeostasis and hormonal responsiveness (*not shown*). As two weeks are consistent with protein synthesis and turnover, an indication is there, albeit indirect, that returning cellular protein content to its physiological level is crucial to restore Mg^{2+} buffering and homeostasis as well as β-adrenergic receptor related signaling.

A major difference appears to exist between β-adrenergic receptor-mediated Mg^{2+} extrusion in heart (present study) versus liver [Fagan et al., 2004]. While Mg^{2+} extrusion is inhibited in the heart, the process appears to occur normally in liver cells, irrespective of the time elapsed since diabetes onset. The cellular distribution of Mg^{2+} within the two cell types is comparable, with a cytoplasm Mg^{2+} pool, partially complexed with ATP and phosphonucleotides, and three main pools in the nucleus, mitochondria and endoplasmic (liver) or sarcoplasmic (heart) reticulum. Therefore, it is unlikely that the cellular distribution is responsible for the different response. Most likely, the difference depends on the different β-adrenergic receptor isoform predominantly expressed by the two tissues: β_1 in the cardiac myocytes and β_2 in the hepatocytes [Xiao et al., 1994], and the associated post-receptor signaling [Pavoine et al., 1999]. While β_1-adrenergic receptor signals almost exclusively via adenylyl cyclase and cAMP, β_2-adrenergic receptor signaling is

not restricted to cAMP but involves also Ca^{2+} and/or arachidonic acid [Pavoine et al., 1999] as additional second messengers. It is therefore possible that either messenger, or their combination, has additional and unexplored effects on Mg^{2+} homeostasis in liver cells.

Pathological Significance

The implications of a reduced cellular Mg^{2+} content for the proper functioning of cardiac myocytes can be numerous and far reaching. For one, it has been proposed that Mg^{2+} can modulate the occupancy of troponin C Ca^{2+}-binding pockets under resting conditions, thus regulating how rapidly the contraction process takes place [Sousa et al., 2006]. Hence, a decrease in Mg^{2+} content associated with an increase in cellular Ca^{2+} level can have a major effect on cardiac contractility/relaxation under diabetic conditions. Second, as reticular Ca^{2+} release is inversely proportional to cytoplasm Mg^{2+} level [Duggleby et al., 1999], Ca^{2+} cycling between cytoplasm and sarcoplasmic reticulum can be expected to be enhanced in diabetic myocytes. Third, specific mitochondrial dehydrogenases are particularly sensitive to matrix Mg^{2+} level, their activity being reduced under conditions in which mitochondrial Mg^{2+} content is decreased [Panov and Scarpa, 1996]. Fourth, a decrease in cellular Mg^{2+} content can affect protein synthesis, as mentioned previously. Lastly, a decrease in cellular Mg^{2+} level has been correlated to an increase in lipid peroxidation [Gunther et al., 1995]. Previous observations from our laboratory indicate that cardiac mitochondria from 2 to 8 weeks diabetic rats, which present a decrease in Mg^{2+} content, undergo lipid peroxidation and generation of 4-hydroxy-nonenal to a higher extent than mitochondria from age-matched non-diabetic rats [Lashin et al., 2006]. Interestingly, also in that study insulin supplementation for 2 weeks abolishes almost completely lipid peroxidation and generation of 4-hydroxynonenal, and restores mitochondrial functions [Lashin et al., 2006].

CONCLUSIONS

In conclusion, our results indicate that cardiac cells undergo a considerable Mg^{2+} loss as a result of type-I diabetes onset. The Mg^{2+} loss is associated with a decrease in protein and ATP level within the myocytes, which ultimately compromises the physiological functioning of cardiac cells. Two weeks of insulin treatment are necessary to restore Mg^{2+} homeostasis and transport to its physiological levels. Our observation provides an important framework for further studies aimed at understanding the role of Mg^{2+} loss for altered cardiac functions and short- and long-term diabetic complications in cardiac tissue.

ACKNOWLEDGMENTS

This study was in part supported by Howard Hughes Summer Fellowship to Mr. Grant Reed, and by NIH-HL18708 and NIH-AAA AA11593 to Dr. Andrea Romani.

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