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Adenosine triphosphate depletion by cyanide results in a Na^+-dependent Mg^{2+} extrusion from liver cells

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Abstract

Addition of NaCN to isolated hepatocytes results in a marked and rapid decrease in cellular adenosine triphosphate (ATP) content, and in the extrusion of a sizable amount of cellular Mg^{2+}. This extrusion starts after a 10-minute lag phase and reaches a maximum of 35 to 40 nmol Mg^{2+} per milligram protein within 60 minutes from the addition of CN^-. A quantitatively similar Mg^{2+} extrusion is also observed after the addition of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone but not that of the glycolysis inhibitor iodoacetate. The Mg^{2+} extrusion is completely inhibited by the removal of extracellular Na^+ or the addition of imipramine, quinidine, or glibenclamide, whereas it persists after the removal of extracellular Ca^{2+} or K^+, or the addition of amiloride. An acidic extracellular pH or the removal of extracellular HCO_3^- inhibits the cyanide-induced Mg^{2+} extrusion by at least 80%. Taken together, these data suggest that the decrease in cellular adenosine triphosphate content removes a major Mg^{2+} complexing agent from the hepatocyte and results in an extrusion of hepatic Mg^{2+} exclusively through a Na^+-dependent exchange mechanism modulated by acidic changes in extracellular pH.

1. Introduction

After potassium, magnesium (Mg^{2+}) is the second most abundant cation within mammalian cells [1,2]. The majority of cellular Mg^{2+} is compartmentalized within nucleus, mitochondria, and endosarcoplasmic reticulum [3], with concentrations ranging between 16 and 20 mmol/L in each of these compartments. Approximately 20% of total cellular Mg^{2+} content is in the cytoplasm, in the form of a complex with adenosine triphosphate (ATP) (~4-5 mmol/L) and, to a lesser extent, other phosphonucleotides and cytosolic proteins [3]. As a result of this distribution, cytosolic free Mg^{2+} concentration is estimated to be approximately 0.7 mmol/L Mg^{2+} or less than 4% of total cellular content [3]. Although total and free cellular Mg^{2+} content does not change significantly under resting conditions, major fluxes of Mg^{2+} can cross the cell membrane in either direction as a result of hormonal or metabolic stimuli (reviewed by Romani [4] and Wolf et al [5]). For the most part, these fluxes result in detectable changes in total cellular Mg^{2+} content, whereas cytoplasm *free* [Mg^{2+}] changes minimally, if at all [6].

The extrusion of Mg^{2+} across the cell membrane occurs via the distinct operation of a Na^+-dependent and a Na^+-independent mechanism [7]. In the absence of functional cloning and more precise structural information, the Na^+ dependent Mg^{2+} extrusion mechanism has been tentatively identified as a Na^+/Mg^{2+} exchanger based upon its strict requirement for extracellular Na^+ to operate [8] and its inhibition by nonspecific Na^+-transport inhibitors such as amiloride, quinidine, or imipramine (see Gunther [7] as a review). This exchanger is activated by the increase in cytosolic cyclic adenosine monophosphate (cAMP) level that follows the stimulation of β-adrenergic, glucagon, or prostaglandin receptors as well as the addition of forskolin or cell-permeant cAMP analogs [5,9]. Under conditions in which the operation of this exchanger is inhibited, Mg^{2+} can still be extruded from the cell via a not well-characterized Na^+-independent mechanism [10] that uses various cations or anions [7] to favor Mg^{2+} efflux. The operation of these 2 distinct transport mechanisms in the hepatocyte membrane has been confirmed by our laboratory in purified liver plasma membrane vesicles [11]. Furthermore, our group has located the operation of the Na^+-dependent and Na^+-independent

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mechanism in the basolateral and the apical domain of the hepatocyte membrane, respectively [11], with only the Na^+-dependent mechanism specifically requiring a cAMP-dependent phosphorylation to operate [12].

As more than 80% of cytoplasmic Mg^{2+} forms a complex with ATP, expectation is there that a marked decrease in ATP content will result in a proportional increase in cytoplasmic free [Mg^{2+}]. Experiments performed in hepatocytes [13] and Jurkat cells [14], however, indicate that after addition of cyanide, cellular ATP decreases markedly and rapidly, whereas cytosolic free Mg^{2+} increases only minimally [15] or not at all [14]. Therefore, possibility is there that cytosolic Mg^{2+} is either extruded from the cell or compartmentalized within organelles as a result of a change in cellular ion pattern. The decrease in ATP content can result in a decrease in the activity of the Na^+/K^+-ATPase [16] and in the activation of K_{ATP} channels [17,18], a class of plasma membrane channels modulated by ATP and Mg^{2+}. The release of K^+ through this channel would counterbalance the entry of Na^+ that occurs via Na^+/H^+ antiporter [19] and/or Na^+/HCO_3^- cotransporter [20] after a marked cellular acidification as that observed under chemical hypoxia conditions [20].

Hence, the present study was undertaken (1) to test the hypothesis that the lack of an increase in cytosolic free Mg^{2+} after a cyanide-induced decrease in cellular ATP level depends on an extrusion of Mg^{2+} from the cytoplasm into the extracellular space and (2) to determine the relative role of the Na^+-dependent and the Na^+-independent transport mechanisms in mediating such an extrusion. The results reported here indicate that, after cyanide administration, Mg^{2+} is extruded from liver cells exclusively through a Na^+-dependent mechanism highly responsive to acidic changes in extracellular pH.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents were from Sigma (St Louis, MO). Collagenase type II was from Worthington (Freehold, NJ). Pinacidil and glibenclamide were from ICN Radiochemicals (Costa Mesa, CA).

2.2. Animals

Animal handling and experimental utilization conformed to the National Institutes of Health guidelines for the care and use of laboratory animals, and the relative protocol was approved by Case Western Reserve University Institutional Animal Care and Use Committee.

2.3. Cell Isolation

Male Sprague-Dawley rats (200-250 g body weight) were anesthetized by intraperitoneal injection of sature sodium pentobarbital solution (50 mg/kg body weight). After induction of deep anesthesia, the abdomen was incised and the portal vein was cannulated. Collagenase dispersed hepatocytes were isolated according to the procedure of Seglen as reported previously [21]. After isolation, cells were resuspended in a buffer containing the following (in millimoles per liter): 120 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, 12 $NaHCO_3$, and 10 HEPES (pH 7.2), in the presence of O_2:CO_2 (95:5 vol/vol). Trypan blue exclusion test indicated that the hepatocytes viability was 85% ± 3% (n = 9) immediately after isolation and did not change significantly over the 4 hours of our experiment (84% ± 5%, n = 9).

2.4. Determination of Mg^{2+} transport

Hepatocytes were washed twice in a medium similar to the one described above but devoid of Mg^{2+} (*Mg^{2+} free buffer*), and incubated therein, at the final concentration of 0.3 to 0.4 mg protein per milliliter, at 37°C, under continuous stirring and O_2:CO_2 flow (95:5 vol/vol). The amount of Mg^{2+} present as contaminant in the medium was measured by atomic absorbance spectrophotometry (AAS) in a Perkin-Elmer 3100 (Perkin Elmer, Waltham, MA) and found to be less than 3 μmol/L. At selected time points, 0.7-mL aliquots of the incubation mixture were withdrawn in duplicates and rapidly sedimented in microcentrifuge tubes at 1200 rpm for 5 minutes. The supernatants were removed, and their Mg^{2+} content was determined by AAS. The cell pellets were digested overnight in 0.7 mL of 10% HNO_3. After sedimentation of denatured protein at 14 000 rpm for 5 minutes in microfuge tubes, the Mg^{2+} content of the acid extracts was measured by AAS.

For the experiments in a Na^+-free medium, NaCl and $NaHCO_3$ were replaced with equimolar concentrations of choline chloride and $KHCO_3$, respectively. Potassium hydroxide was used to attain pH 7.2. For the experiments in K^+-free or Ca^{2+}-free medium, these cations were simply removed from the incubation medium.

2.5. Determination of cytosolic free Mg^{2+} concentration

Changes in cytosolic free Mg^{2+} concentration ($[Mg^{2+}]_i$) were measured by Mag-Fura2 (Molecular Probe, Portland, OR) as described previously [6]. Hepatocytes were loaded with the cell-permeant fluorescent indicator Mag-Fura2-AM (10 μmol/L) for 20 minutes at room temperature to prevent entrapment of the dye within cellular organelles. Hepatocytes were then washed to remove excess dye and transferred into a thermostated cuvette (37°C), under continuous stirring, using a custom-built dual-wavelength excitation fluorimeter (University of Pennsylvania Biomedical Instrumentation Group, Philadelphia, PA) equipped with narrow-bandwidth interference filters. The excitation wavelengths for the dual-wavelength fluorimeter were 340 and 380 nm, respectively, whereas the emission wavelength was 510 nm. The Mg^{2+} concentration was calculated using the following formula: $[Mg^{2+}]i = Kd * [(R - Rmin)/(Rmax - R)] * (Sf/Sb)$,

where *K*d = 1.2 mmol/L [6,22]. Hepatocytes were incubated either in the presence or in the absence of extracellular Na^+ using the buffer compositions described previously. Because of the unreliability in obtaining Mag-Fura2 *R*max and *R*min in situ, calibration of changes in the fluorescence dual-wavelength signal ratio was conducted using a cytosol-like buffer C containing (in millimoles per liter) 130 KCl, 10 NaCl, 1.2 KH_2PO_4, 10 HEPES, and 0.5 $MgCl_2$ at pH 7.2 and an amount of nonesterized Mag-Fura2 yielding, at the isosbestic point of 360 nm, a fluorescence quantitatively similar to that measured in Mag-Fura2-AM loaded cells. *R*max was determined by adding known aliquots of $MgCl_2$ until maximal fluorescence was reached. *R*min was determined by adding increments of EDTA until all Mg^{2+} was complexed [6,22].

2.6. ATP measurement

Cellular ATP content was measured by luciferin-luciferase assay [23]. Aliquots of the incubation mixtures were withdrawn at selected time points and sedimented in microfuge tubes. The Mg^{2+} content of the supernatant was measured by AAS as described previously. The cell pellets were digested with 5% PCA for 5 minutes on ice. The acid mixture was neutralized by addition of 0.7 mL of 1 mol/L $KHCO_3$ and centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatants were removed and stored at −20°C until used. The ATP content in the supernatant was measured by luciferin-luciferase assay (Sigma kit with a detecting sensitivity in the picomole-nanomole per milliliter range) using a LUMAT Berthold LB 9501 luminometer (Berthold, Huntsville, AL).

2.7. Protein measurement

The protein was measured according to the procedure of Bradford [24].

2.8. Statistical analysis

Data are presented as means ± SE. Data were analyzed by 1-way analysis of variance. Multiple means were then compared by Tukey multiple comparison test performed with a level of statistical significance designated as $P < .05$.

3. Results

3.1. Mg^{2+} efflux upon chemical anoxia

The addition of various metabolic inhibitors to suspensions of dispersed hepatocytes resulted in a marked release of cellular Mg^{2+} into the extracellular milieu (Fig. 1). The Mg^{2+} release was already detectable 10 minutes after the addition of 2 mmol/L NaCN and increased over time in a time-dependent manner. After 20 minutes from CN^- addition, 16.2±1.2 nmol Mg^{2+} per milligram protein has been released in the extracellular medium. Maximal extrusion was achieved at t = 60 minutes from the agent administration

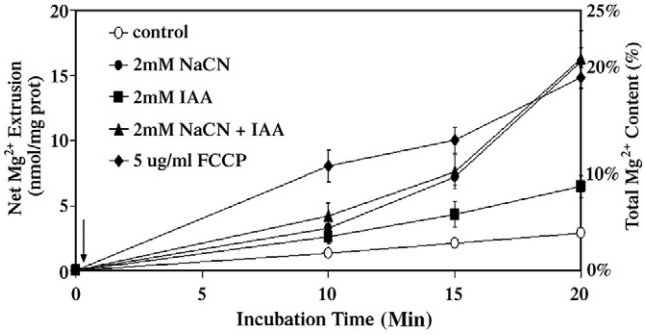

Fig. 1. Mg^{2+} extrusion from liver cells upon induction of chemical hypoxia. Hepatocytes were isolated and incubated as reported in "Materials and methods." Cells were stimulated by addition of 2 mmol/L NaCN (CN^-), 2 mmol/L iodoacetic acid (IAA), NaCN plus IAA, or 5 μg/mL FCCP (arrow). The amount of Mg^{2+} extruded from the cells is reported as the nanomoles Mg^{2+} per milligram protein and as a percentage of the total cellular Mg^{2+} content. Data are means ± SE of 6 different preparations, each assessed in duplicate. All the data points relative to hepatocytes treated with NaCN, FCCP, IAA, and NaCN + IAA are statistically significant vs corresponding values in control samples. Labeling is omitted for simplicity.

(36.6±1.1 nmol Mg^{2+} per milligram protein). Addition of the mitochondrial uncoupler *p*-trifluoromethoxy-phenylhydrazone (FCCP) (5 μg/mL) also resulted in a marked extrusion of Mg^{2+} from the hepatocytes. In the presence of the uncoupler, however, the amplitude of Mg^{2+} extrusion was enhanced at all the time points, although it reached an end point similar to that measured in the presence of NaCN (Fig. 1). By contrast, Mg^{2+} extrusion elicited by the addition of the glycolysis inhibitor iodoacetate (2 mmol/L) was comparatively smaller (2.62 ± 0.9 nmol/mg protein per 10 minutes) (Fig. 1). Larger doses of iodoacetate (up to 5 mmol/L) did not result in a significant enlargement of Mg^{2+} extrusion (not shown). The amount of Mg^{2+} released into the extracellular milieu at t = 20 minutes after the

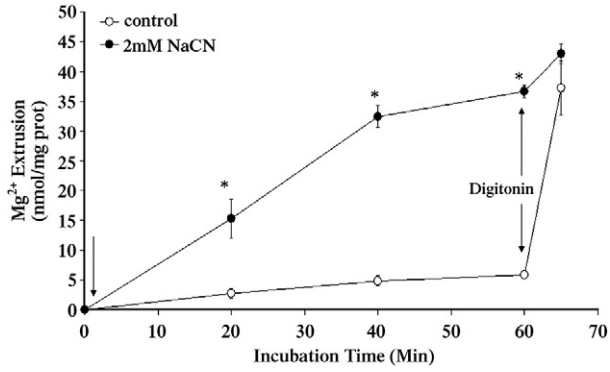

Fig. 2. Digitonin-releasable Mg^{2+} from liver cells treated with NaCN. Hepatocytes were isolated and incubated as reported in "Materials and methods." Cells were treated with digitonin (50 μg/mL) after 40 minutes of stimulation with 2 mmol/L NaCN (first arrow). The amount of Mg^{2+} extruded from the cells is reported as the nanomoles Mg^{2+} per milligram protein. Data are means ± SE of 6 different preparations, each assessed in duplicate. *Statistically significant vs corresponding value in NaCN-untreated cells.

addition of CN^- or FCCP (16.7 nmol Mg^{2+} per milligram protein) was comparable to the decrease in Mg^{2+} content in the cell pellets (15.4 ± 0.8 nmol Mg^{2+} per milligram protein, n =7) and accounted for approximately 20% of total cellular Mg^{2+} (Fig. 1). A similar correlation was observed in hepatocytes treated with CN^- for 60 minutes (data not shown).

Addition of 50 μg/mL digitonin at t = 60 minutes after CN^- addition resulted in the release of a small residual amount of Mg^{2+} from the hepatocytes (Fig. 2). In contrast, addition of digitonin to control hepatocytes released more than 30 nmol Mg^{2+} per milligram protein, reaching an end point that was not statistically different from that obtained in CN^--treated cell (Fig. 2). Digitonin addition also induced the release of a significant amount of ATP from control hepatocytes (25.3 ± 3.7 nmol ATP per milligram protein), but only 1.8 ± 0.6 nmol ATP per milligram protein from NaCN-treated cells (n = 5 for both experimental conditions, $P < .001$).

Under these experimental conditions, cytosolic free $[Mg^{2+}]i$ changed transiently as a result of CN^- addition (Fig. 3). A net increase equivalent to 0.4 ± 0.08 mmol/L was observed at time 10 minutes after CN^- addition (Fig. 3A, trace a, and Fig. 3B). This increase in concentration declined toward basal level by time = 20 minutes from CN^- addition

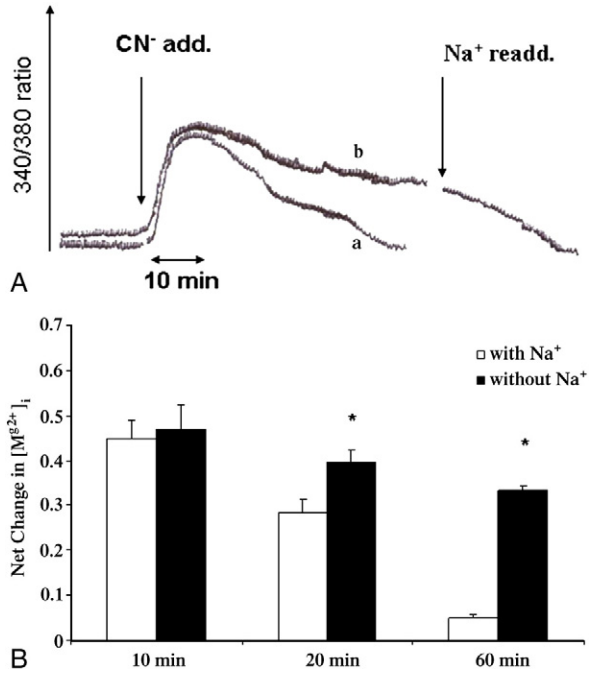

Fig. 3. Changes in cytosolic free $[Mg^{2+}]i$. Changes in cytosolic free Mg^{2+} concentration were determined as described in detail in "Materials and methods." Hepatocytes were incubated both in the presence (trace a) and in the absence (trace b) of extracellular Na^+. A, A typical experiment out of 6 different preparations for both experimental conditions, each tested in duplicate. B, The net change in cytosolic free $[Mg^{2+}]i$ at time 10, 20, and 60 minutes after CN^- addition for hepatocytes incubated in the absence and in the presence of extracellular Na^+. Data are means ± SE of 6 different preparations, each tested in duplicate for both experimental conditions. *Statistically significant vs value in the presence of extracellular Na^+.

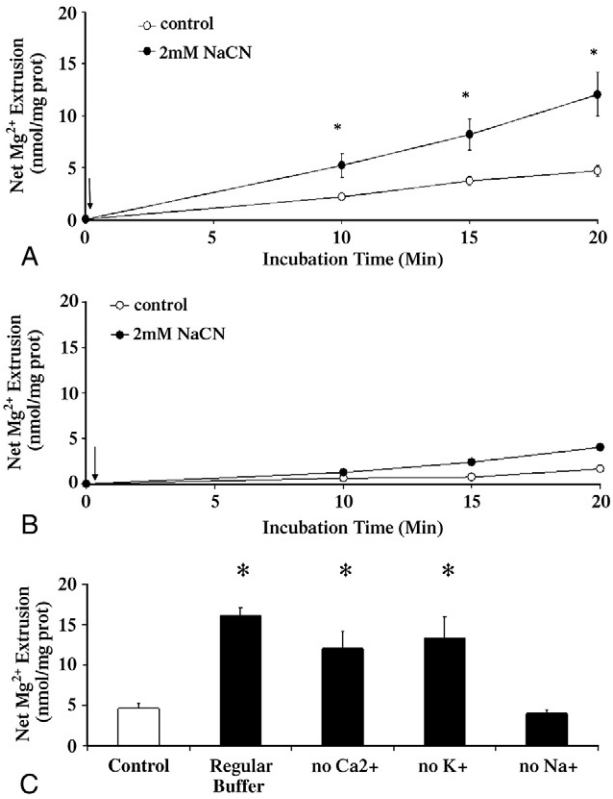

Fig. 4. Na^+-dependence of Mg^{2+} extrusion from liver cells upon induction of chemical hypoxia. Hepatocytes were isolated and incubated as reported in "Materials and methods." Cells were incubated in a medium devoid of extracellular Ca^{2+} (A), K^+ (not shown), or Na^+ (B), and treated with NaCN (arrow = time of addition). C, Net amount of Mg^{2+} released in the extracellular medium upon addition of 2 mmol/L NaCN is reported as nanomoles per milligram protein. Data are means ± SE of 5 different preparations, each assessed in duplicate. *Statistically significant vs corresponding value in control cells and cells treated with NaCN in the absence of extracellular Na^+.

(0.1 ± 0.03 mmol/L), remaining at these levels for the remainder of our experimental protocol (Fig. 3A, trace a, and Fig. 3B). When similar experiments were performed on hepatocytes incubated in the absence of extracellular Na^+, cytosolic free $[Mg^{2+}]_i$ remained elevated for the duration of the experiment (Fig. 3A, trace b, and Fig. 3B) until extracellular Na^+ was reintroduced to the system (Fig. 3A).

Table 1
Cell viability after addition of cyanide or FCCP in the absence or in the presence of extracellular Na^+

	0 min	10 min	20 min
Experimental conditions			
Control (with Na^+)	82.9 ± 3.2	81.0 ± 4.2	78.9 ± 5.7
2 mmol/L NaCN (with Na^+)	82.2 ± 2.8	83.4 ± 3.7	77.7 ± 6.1
5 μg/mL FCCP (with Na^+)	83.2 ± 3.3	81.9 ± 4.5	79.5 ± 5.2
Control (without Na^+)	81.8 + 4.4	84.5 ± 2.8	80.7 ± 4.7
2 mmol/L NaCN (without Na^+)	85.6 ± 2.3	82.3 ± 4.6	82.7 ± 2.8

Cell viability was assessed by trypan blue exclusion test. Values are percentage of cells trypan blue negative at the indicated time points. Data are means ± SE of 5 different experiments, each performed in duplicate.

To exclude that Mg^{2+} extrusion resulted from a nonspecific decrease in permeability, trypan blue exclusion test and determination of lactate dehydrogenase (LDH) release were performed on aliquots of cell suspension. As Table 1 shows, between 78% and 82% of CN^--treated cells (down from the initial 84%-85%) remained trypan blue negative after 20 minutes of incubation in the absence or in the presence of CN^-, irrespective of the absence or the presence of extracellular Na^+. No significant release of cytosolic LDH was observed during this period (not shown), further excluding the occurrence of cell death or a nonselective increase in plasma membrane permeability. To minimize possible inconsistencies in our Mg^{2+} determinations due to variations in the number of dead hepatocytes upon metabolic treatment, for the remainder of our study, we primarily focused on the 20-minute time point.

3.2. Dependence of Mg^{2+} extrusion on extracellular Na^+

Previous observation from this and other laboratories indicates that cellular Mg^{2+} is extruded across the plasma membrane of liver cells through a Na^+-dependent and a Na^+-independent mechanism under a variety of hormonal stimuli or experimental conditions [4,5,7,25,26]. The possibility that the cyanide-induced Mg^{2+} extrusion occurred via either mechanism was investigated by selectively modifying the cation composition of the extracellular medium [27]. Removal of extracellular Ca^{2+} (Fig. 4A) or K^+ (not shown) had little or no effect on the amplitude of Mg^{2+} extrusion induced by CN^-. Removal of extracellular Na^+, instead, abolished Mg^{2+} extrusion almost completely (Fig. 4B) as already suggested by the persistent

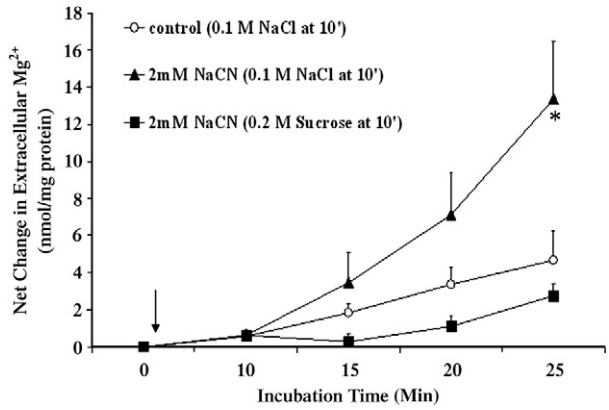

Fig. 5. Effect of Na^+ reintroduction on Mg^{2+} extrusion from liver cells treated with NaCN. Hepatocytes were isolated and incubated in medium devoid of Na^+ as reported in "Materials and methods." After administration of 2 mmol/L NaCN, or 2 mmol/L NaCN plus 2 mmol/L iodoacetate, 100 mmol/L NaCl, 100 mmol/L KCl, or an equiosmolar concentration (200 mmol/L) of sucrose was added to the incubation system at time = 10 minutes. Data are means ± SE of 5 different preparations, each assessed in duplicate for each experimental condition, and are expressed as nanomoles Mg^{2+} per milligram protein. *Statistically significant vs corresponding value in control cells.

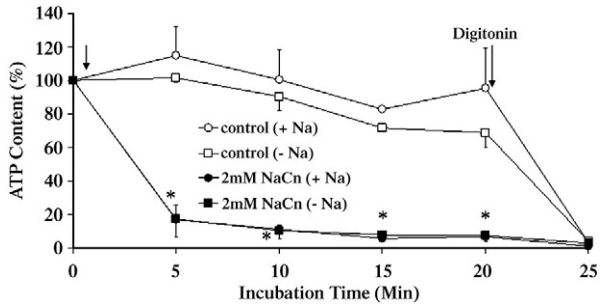

Fig. 6. Change in cellular ATP content upon addition of NaCN. Hepatocytes were isolated and incubated in the absence or in the presence of extracellular Na^+ and stimulated by addition of 2 mmol/L NaCN (first arrow) as reported in "Materials and methods." Cellular ATP content was assessed by luciferin-luciferase assay as reported previously [23]. Data are means ± SE of 5 different preparations, each assessed in duplicate. *Statistically significant vs corresponding value in control cells.

elevation in cytosolic free $[Mg^{2+}]i$ (Fig. 3). The net amount of Mg^{2+} mobilized into the extracellular medium under the various experimental conditions is reported in Fig. 4C. The selective dependence of Mg^{2+} extrusion on extracellular Na^+ was further supported by the observation that reintroduction of a physiologic concentration of extracellular Na^+ at a later time point after CN^- addition restored the mobilization of Mg^{2+} into the extracellular compartment (Fig. 5), as indicated by the decrease in intracellular $[Mg^{2+}]_i$ (Fig. 3A, trace b). To exclude that the Mg^{2+} extrusion observed after Na^+ reintroduction was due to an osmolarity mismatch, an equiosmolar concentration of sucrose (Fig. 5) or KCl (not shown) was added to the medium instead of the indicated concentration of NaCl. Neither sucrose nor KCl addition was able to elicit Mg^{2+} extrusion from the hepatocytes.

3.3. ATP content

The effectiveness of NaCN in decreasing cellular ATP content was assessed. As Fig. 6 shows, hepatic ATP content decreased to less than 20% of the initial value within 5

Table 2
Mg^{2+} extrusion after addition of 2 mmol/L NaCN, in the presence of amiloride, quinidine, imipramine, and glibenclamide

Experimental conditions	nmol Mg^{2+}/(mg protein 20 min)
Control	1.7 + 0.7
2 mmol/L NaCN	16.7 ± 1.2*
NaCN plus 1 mmol/L amiloride	18.3 ± 3.8*
NaCN plus 1 mmol/L quinidine	4.6 ± 0.5
NaCN plus 100 μmol/L imipramine	4.4 ± 0.2
NaCN plus 100 μmol/L glibenclamide	5.5 ± 0.3
NaCN plus 100 μmol/L Pinacidil	17.6 ± 1.9*

Data are means ± SE of 5 different experiments, each performed in duplicate.

* $P < .001$ as compared with control value and with Mg^{2+} extrusion elicited by NaCN in the presence of quinidine, imipramine, or glibenclamide.

Table 3
Dependence of NaCN-induced Mg^{2+} extrusion on extracellular pH

	nmol Mg^{2+}/(mg protein 20 min)
Experimental conditions	
pH = 7.2	16.7 ± 1.2
pH = 6.5	3.4 ± 0.9*
pH = 8.0	21.3 ± 4.7†
without HCO_3^-	6.0 ± 1.5*

Data are means ± SE of 5 different experiments, each performed in duplicate.
* $P < .11$ as compared with the pH 7.2 value.
† $P < .45$ as compared with the pH 7.2 value.

minutes from the administration of NaCN irrespective of the absence or the presence of Na^+ in the extracellular milieu. In contrast, ATP level remained relatively stable in untreated cells throughout the duration of the experiment irrespective of the absence or the presence of extracellular Na^+.

3.4. Pharmacologic inhibition of Mg^{2+} extrusion

Presently, no specific inhibitor of the putative Na^+/Mg^{2+} exchanger is available. Mg^{2+} extrusion, however, can be effectively blocked by amiloride, imipramine, or quinidine in cells (see Harman et al [13] as a review) as well as in liver plasma membrane vesicles [11]. As Table 2 shows, the addition of amiloride up to 1 mmol/L did not block the extrusion of Mg^{2+} induced by cyanide (Table 2). By contrast, 100 μmol/L imipramine or 1 mmol/L quinidine, 2 agents able to block a variety of Na^+ transport mechanisms including Na^+ channels as well as K^+ channels, inhibited to a comparable extent (≥75%) the extrusion of Mg^{2+} induced by cyanide. Experimental evidence indicates that K_{ATP} channels are activated by the decrease in cellular ATP content subsequent to chemical hypoxia [17,18]. To test the possibility that quinidine and imipramine prevented Mg^{2+} extrusion by blocking the K_{ATP} channels, glibenclamide [18,28] and pinacidil [29] were used as specific inhibitor and agonist of these channels, respectively. As Table 2 shows, 10 μmol/L glibenclamide inhibited the extrusion of Mg^{2+} induced by cyanide to an extent comparable to that observed in the presence of quinidine and imipramine. By contrast, pinacidil did not elicit an extrusion of Mg^{2+} per se; nor was it effective at potentiating the extrusion elicited by cyanide administration.

3.5. pH regulation of Mg^{2+} extrusion

Chemical hypoxia is characterized by a marked decrease (~ 0.35 units) in intracellular pH [30]. An acidic intracellular pH affects the affinity constants of Mg^{2+} for binding components, mainly phosphonucleotides [30], and results in modest increase in cytosolic free $[Mg^{2+}]_i$ [13,31]. On the other hand, acidic extracellular pH has been shown to protect isolated cells and perfused organs from the deleterious effects of anoxia by limiting the entry of Na^+ into the cells [20]. Hence, we investigated whether changes in extracellular pH could modulate the amplitude of Mg^{2+} extrusion. The results reported in Table 3 indicate that either extracellular acidic pH or removal of HCO_3^- from the incubation medium inhibited by approximately 80% the amplitude of Mg^{2+} extrusion. Incubation in an alkaline medium (pH 8.0) tended to enlarge the amplitude of Mg^{2+} extrusion, but the trend did not attain statistical significance.

4. Discussion

Cellular Mg^{2+} is highly compartmentalized within organelles (viz, mitochondria, endoplasmic or sarcoplasmic reticulum, and nucleus [3,32]) and cytoplasm, in which it forms a complex predominantly with ATP [30,33] but also other phosphonucleotides and proteins [33]. The rapid and massive decrease in cytosolic ATP content induced by cyanide or other mitochondria metabolic inhibitors results in a rise in cytosolic free $[Mg^{2+}]$ that is far slower and smaller than it would be expected based upon the rate and amplitude of ATP decrease [30,34]. Circumstantial evidence has suggested the possibility that the increase in cytosolic free $[Mg^{2+}]$ induced by chemical hypoxia is sufficiently high to displace Ca^{2+} from its relatively few cytosolic binding sites [30], especially in liver cells. However, the amount of Ca^{2+} that can be displaced from the hepatocyte's cytosol is rather limited [30]. Hence, additional mechanisms must be invoked to explain the small rise in cytosolic free Mg^{2+} observed under these conditions.

In the present study, the possibility that the limited increase in cytosolic free Mg^{2+} observed upon chemical hypoxia and ATP degradation was due at least in part to a Mg^{2+} extrusion across the hepatocyte plasma membrane was investigated.

4.1. Mg^{2+} extrusion

After the addition of cyanide, or FCCP, the cellular level of ATP decreased by 80% to 85% within 5 minutes. By contrast, a small but detectable release of Mg^{2+} from the cell became evident only after a lag phase of 10 minutes and increased progressively over time to reach the maximum after approximately 60 minutes from the time of the agent addition. At this later time point, the addition of digitonin failed to mobilize a significant amount of additional Mg^{2+} from the cytoplasm, indicating that all cytosolic Mg^{2+} had already been extruded. Assessment of Mg^{2+} content within mitochondria and endoplasmic reticulum indicated that these organelles retained the same amount of Mg^{2+} irrespective of the addition of CN^- in the absence or in the presence of external Na^+ (data not shown). Hence, these data exclude the possibility that Mg^{2+} redistribution among cytoplasm and cellular organelles occurred under our experimental conditions, and further emphasize that Mg^{2+} extrusion is the predominant cause of Mg^{2+} loss from the cytoplasm. At the present time, we do not have an explanation for the observed lag phase in Mg^{2+} extrusion. One possibility is that a certain increase in cytosolic free $[Mg^{2+}]i$ has to be attained before

the cell "senses" the increase and activates the Mg^{2+} extrusion mechanisms. Because the nature of this "sensor" is still undetermined, we are not in a position to properly test this hypothesis. An alternative explanation is that the lag phase represents an attempt by the cell to buffer the excess free Mg^{2+} through cytosolic proteins or adenosine diphosphate and AMP generated from the degradation of ATP. As the levels of adenosine diphosphate and AMP also decline overtime after CN^- poisoning, increased mobilization and extrusion of Mg^{2+} from the cell would result. The profile of the changes in cytosolic free $[Mg^{2+}]i$, however, would be consistent with the first of these possibilities, as a larger increase in $[Mg^{2+}]i$ was recorded at a time point after CN^- addition at which little Mg^{2+} extrusion was detected in the extracellular space (compare Figs. 1 and 3). As for the different extrusion profile observed after CN^- or FCCP addition, this could be explained by the marked lipophilicity of FCCP, which results in the rapid redistribution of the uncoupler within the mitochondrial membrane and collapses the proton gradient across the mitochondrial membrane. It has to be noted, however, that the end point for both CN^- and FCCP-induced Mg^{2+} extrusion is quite similar (Fig. 1), suggesting the draining of the same pool.

The release of Mg^{2+} from the hepatocyte is a very specific process as attested by the following lines of evidence: (1) Mg^{2+} extrusion occurs in the absence of LDH release or changes in cell membrane permeability, cell viability, or extracellular osmolarity; (2) Mg^{2+} is extruded from the cell via the operation of a transport mechanism that is blocked by agents that inhibit Na^+ transport (although not specifically), or by the removal of extracellular Na; and (3) reintroduction of Na^+ under the latter condition is essential to restore Mg^{2+} extrusion to an extent comparable to that observed under normal experimental conditions.

Taken together, these results are consistent with the activation of a specific transport mechanism which, based upon its strict Na^+ dependence, can be tentatively identified with the Na^+/Mg^{2+} exchanger operating in various cell types including hepatocytes [1,7,8]. Our results are also consistent with numerous experimental observations including work by Almulla et al [35] and Kubota et al [25,26] with fluorescent indicators indicating a persistent elevation in cytosolic free $[Mg^{2+}]_i$ under conditions in which extracellular Na^+ is not available to favor Mg^{2+} extrusion. The specific inhibition of Mg^{2+} extrusion by imipramine or quinidine, 2 agents reported to block the Na^+-dependent Mg^{2+} transport in a variety of cells [11,36], further indicates that the extrusion of Mg^{2+} induced by the addition of cyanide occurs through a specific transport mechanism. Surprisingly, amiloride, the drug most commonly used to inhibit the Na^+-dependent Mg^{2+} extrusion from liver and other tissues [7] under various experimental conditions, appears to be ineffective at inhibiting the cyanide-induced Mg^{2+} extrusion even when administered at millimoles-per-liter concentrations. The reason for this lack of an effect is not clear. At least 3 explanations are at hand. The first possibility is that amiloride only inhibits Mg^{2+} transport under conditions in which cellular ATP content is not decreased or is only marginally affected by treatment. Experiments in purified total liver plasma membrane devoid of ATP, however, indicate that this is not the case in that amiloride and imipramine are equally effective at inhibiting Na^+-dependent Mg^{2+} extrusion [11]. A second possibility is that imipramine (or quinidine) and amiloride inhibit different Na^+-dependent Mg^{2+} extrusion mechanisms. This possibility is supported by the experimental evidence that, in purified liver plasma membrane vesicles, amiloride only inhibits a Na^+/Mg^{2+} exchange mechanism present in the apical domain (bile side) of the hepatocyte, leaving unaffected the one operating in the basolateral domain (blood side) of the cell [11]. In contrast, imipramine inhibits both exchangers to a comparable extent [11]. This observation could explain the results reported here as well as why the coaddition of imipramine and amiloride does not exert an additive inhibitory effect on the Na^+-dependent Mg^{2+} extrusion from intact hepatocytes (Romani, personal observation) or purified liver plasma membranes even in the absence of ATP [37]. A third and not mutually exclusive explanation would be that Na^+ is entering the cells through a route insensitive to amiloride. The marked intracellular acidification induced by cyanide treatment [30] activates the Na^+/H^+ antiporter and the Na^+/HCO_3^- sinporter [20] in the attempt to restore cytosolic pH. Whereas the Na^+/H^+ antiporter is inhibited by amiloride [38], the Na^+/HCO_3^- sinporter is amiloride insensitive [39] and can transport Na^+ inwardly even in the presence of high concentrations of the inhibitor. As the accumulated Na^+ cannot be extruded via the Na^+/K^+ ATPase for lack of ATP, Mg^{2+} and possibly K^+ [40,41] would be extruded from the cell for charge compensation.

The observed inhibitory effect of glibenclamide would be consistent with the latter scenario. The plasma membrane of several cells [29] possesses a class of channels regulated by ATP and Mg^{2+} that extrude K^+ when active. These channels, known as *K_{ATP} channels*, become active after the decrease in cellular ATP content induced by metabolic inhibitors [18]. The selective block of these channels by glibenclamide would prevent K^+ extrusion and limit, in turn, the entry of Na^+ into the cells. The end result will be a decrease in the driving force required for the extrusion of Mg^{2+} to occur. It has to be noted that the K_{ATP} channel agonist pinacidil is unable to induce a Mg^{2+} extrusion in the absence of cyanide and does not enhance the cation extrusion induced by the mitochondrial inhibitor. The most likely explanation for these results is that, in the absence of cyanide, the opening of the K_{ATP} channels and the K^+ extrusion do not result in a major entry of Na^+ because of the compensatory activity of the Na^+/K^+-ATPase. Under these conditions, cellular ATP content remains a valid Mg^{2+} buffering system, thus preventing Mg^{2+} mobilization from the cell. After cyanide poisoning and the decrease in cellular ATP content, the activation of K_{ATP} channels and the mobilization of Mg^{2+}

would be already maximal, de facto rendering the effect of pinacidil undetectable.

4.2. Role of pH

One consequence of the decrease in cellular ATP induced by cyanide is the marked cellular acidification [30]. This acidification results from the compensatory activation of the Na^+/H^+ exchanger to remove the excess cellular Na^+ that cannot be extruded via the Na^+/K^+-ATPase because of the decrease in ATP level [30]. This cellular acidification, however, has a major impact on cytosolic Mg^{2+} homeostasis in that it changes the affinity constant of ATP, adenine phosphonucleotides, and phosphocreatine for Mg^{2+} [30] at a time in which the content of these complexing moieties is decreasing as a result of cyanide poisoning. The final result is a facilitated release of Mg^{2+} from its complexes and extrusion from the cell. As indicated by the results reported in Table 3, the difference in transmembrane pH has a major role in regulating cellular Mg^{2+} extrusion. By decreasing the pH difference across the cell membrane, an extracellular acidic pH would reduce the activation of Na^+/H^+ antiporter and Na^+/HCO_3^- sinporter and consequently decrease the entry of Na^+ that constitutes the driving force for Mg^{2+} extrusion to occur [1]. Similarly, the absence of external HCO_3^- would prevent the entry of Na^+ via the Na^+/HCO_3^- sinporter and hamper the amplitude of Mg^{2+} extrusion by affecting the driving force required for its transport. This would imply that not the Na^+/H^+ antiporter but rather the Na^+/HCO_3^- sinporter plays a major role in the Na^+-dependent, cyanide-induced Mg^{2+} extrusion. Support to this hypothesis is provided by the evidence that FCCP, which abolishes the H^+ gradient across all biological membranes including the plasma membrane, elicits an extrusion of Mg^{2+} fairly similar to that induced by cyanide. Thus, it is possible that upon cyanide administration the entry of Na^+ occurs mainly via the Na^+/HCO_3^- sinporter, favored by the extrusion of K^+ through the K_{ATP} channels.

During the preparation of this article, a study by Tashiro et al [42] reported results in cardiac myocytes undergoing a series of experiments similar to those described here in hepatocytes. At variance to what was reported here, Tashiro et al observed that KCN-induced intracellular acidosis and decrease in cellular ATP level below the threshold that elicits rigor cross-bridge formation and cell shortening both contribute to inhibit Mg^{2+} efflux from the cardiac myocytes [42]. Based on these results, the authors propose an absolute requirement of ATP for the operation of the Na^+/Mg^{2+} exchanger in cardiac myocytes [42], as already proposed by Ebel et al [43] in red blood cells.

We do not have a clear explanation for the different ATP requirements in cardiac myocytes and hepatocytes. We cannot exclude that spontaneously contracting cells like cardiac myocytes may regulate differently the Na^+/Mg^{2+} exchanger because of its likely involvement in modulating Na^+ and Ca^{2+} homeostasis and, consequently, cell contractility [44]. On the other hand, it has to be noted that the time of exposure to KCN or Na^+ depleted solution reported by Tashiro and Konishi in most experiments (ie, 70-170 minutes [40]) far exceeded the time used in the present study (20 minutes for the bulk of our reported data).

A last question is whether the Mg^{2+} extrusion is simply the result of reduced buffering capacity in the cytosol and increased entry of Na^+ through the plasma membrane, or it can also operate as an intracellular signal. Gaussin and collaborators [45] have reported that the decrease in cytosolic ATP content that follows fructose administration to hepatocytes, although modest as compared with that attained after cyanide poisoning, results in an increase in cytosolic free $[Mg^{2+}]$ sufficient to activate glycogen phosphorylase and promote glycegenolysis and glycolysis for energy production purpose. Thus, if we consider cytosolic Mg^{2+} as an indirect indicator of the metabolic state of the cell, it is possible to envisage a scenario in which the reduction in cellular ATP content induced by cyanide would lead to a rise in cytosolic $[Mg^{2+}]$ that, in turn, may activate anaerobic glycolysis to produce ATP and compensate, or attempt to, for the inability of mitochondria to provide metabolic energy.

4.3. Conclusions

Taken together, the data reported here suggest that, in liver cells, the Na^+/Mg^{2+} exchange mechanism responsible for the extrusion of cellular Mg^{2+} after hormonal stimulation also plays a key role in extruding Mg^{2+} under condition in which the cytosolic concentration of the cation rises sensibly as a consequence of a decrease in cellular ATP buffering capacity.

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