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Effect of Acute and Prolonged Alcohol Administration on Mg^{2+} Homeostasis in Cardiac Cells

Andrea M.P. Romani

Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106

Abstract

Alcoholic cardiomyopathy represents a major clinical complication in chronic alcoholics. Previous studies from our laboratory indicate that acute and chronic exposure of liver cells to ethanol results in a major loss of cellular Mg^{2+} as a result of alcohol oxidation. We investigated whether exposure to ethanol induces a similar Mg^{2+} loss in cardiac cells. The results indicate that chronic exposure to a 6% ethanol-containing diet depleted cardiac myocytes of >25% of their cellular Mg^{2+} content. Acute ethanol exposure, instead, induced a time- and dose-dependent manner of Mg^{2+} extrusion from perfused hearts and collagenase-dispersed cardiac ventricular myocytes. Pretreatment with chloromethiazole prevented ethanol-induced Mg^{2+} loss to a large extent, suggesting a role of ethanol oxidation via cyP4502E1 in the process. Magnesium extrusion across the sarcolemma occurred via the amiloride-inhibited Na^+/Mg^{2+} exchanger. Taken together, our data indicate that Mg^{2+} extrusion also occurs in cardiac cells exposed to ethanol as a result of alcohol metabolism by cyP4502E1. The extrusion, which is mediated by the Na^+/Mg^{2+} exchanger, only occurs at doses of ethanol 0.1%, and depends on ethanol-induced decline in cellular ATP. The significance of Mg^{2+} extrusion for the onset of alcoholic cardiomyopathy remains to be elucidated.

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Address correspondence to: Andrea M.P. Romani, Department of Physiology and Biophysics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, **Telephone:** +1 216 368 1625, **Fax:** +1 216 368 5586, amr5@po.cwru.edu.

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¹Abbreviations

4-MP	4-methyl-pyrazole
cyP4502E1	cytochrome P450-2E1
CMZ	chloromethiazole
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazine
LDH	lactic dehydrogenase
$[Mg^{2+}]_i$	cytoplasmic free magnesium concentration

Keywords

cardiac ventricular myocytes; magnesium extrusion; ethanol; cyP4502E1; amiloride

1. Introduction

Magnesium (Mg^{2+}) is the second most abundant cation within mammalian cells after potassium (Romani & Scarpa, 1992), and it is highly compartmentalized within nucleus, mitochondria, and endoplasmic or sarcoplasmic reticulum (Günther, 1986; Romani & Scarpa, 1992; Wolf, Torsello, Fasanella, & Cittadini, 2003). Within each of these compartments, total Mg^{2+} concentrations ranging between 16 to 20 mM have been measured by different techniques (Günther, 1986; Romani & Scarpa, 1992; Wolf et al., 2003). Due to technical limitations, however, assessment of free versus bound Mg^{2+} partitioning within some of these compartments remains incomplete (Günther, 1986; Romani & Scarpa, 1992; Wolf et al., 2003). A sizable amount of Mg^{2+} (4–5 mM) is also present in the cytoplasm, mostly in the form of a complex with ATP, phosphocreatine, and other phospho-nucleotides (Scarpa & Brinley, 1981). As a result, cytoplasmic free Mg^{2+} concentration ($[Mg^{2+}]_i$)¹ ranges between 0.5 and 1 mM in the majority of mammalian cells, including cardiac myocytes (Romani & Scarpa, 1992; Wolf et al., 2003).

In the absence of hormonal or metabolic stimuli, cellular Mg^{2+} concentration remains relatively stable within cardiac myocytes. Following stimulation of β -adrenergic receptors by catecholamine or isoproterenol, major and rapid extrusion fluxes of Mg^{2+} across the cell sarcolemma have been observed (Romani, Marfella, & Scarpa, 1993; Romani & Scarpa, 1990; Vormann & Günther, 1987), with minimal or no changes in $[Mg^{2+}]_i$ (Fatholahi, LaNoue, Romani, & Scarpa, 2000). The main mechanism responsible for Mg^{2+} extrusion across the cell membrane of cardiac myocytes has been identified as a cAMP-phosphorylated Na^+/Mg^{2+} exchanger both in intact cells (Romani et al., 1993; Romani & Scarpa, 1990; Vormann & Günther, 1987) and sarcolemmal vesicles (Cefaratti & Romani, 2007).

Clinical and experimental data indicate alcohol consumption as one of the main causes of Mg^{2+} loss from liver cells (Romani, 2008). Our group has extensively investigated the mechanisms responsible for the hepatic Mg^{2+} loss and reported that acute ethanol administration causes two distinct effects. On one hand, ethanol inhibits anaerobic glycolysis in a dose-dependent manner, transiently decreasing cellular ATP and reducing its ability to complex cytoplasmic Mg^{2+} (Tessman & Romani, 1998). The consequent increase in cytosolic free Mg^{2+} promotes Mg^{2+} extrusion through the Na^+/Mg^{2+} exchanger (Tessman & Romani, 1998). On the other hand, ethanol inhibits the Mg^{2+} entry mechanism for more than 45 min after alcohol removal (Torres, Konopnika, Berti-Mattera, Liedtke, & Romani, 2010), *de facto* hampering the restoration of proper Mg^{2+} homeostasis. Similar effects occur on prolonged bases in hepatocytes from animals exposed to alcohol in the diet for 3 weeks (Torres, Cefaratti, Berti-Mattera, & Romani, 2009; Young, Cefaratti, & Romani, 2003).

Ethanol administration is deleterious for several tissues, including the heart, with alcoholic cardiomyopathy representing a major pathological complication in alcoholics (Lucas,

Brown, Wassef, & Giles, 2005). A full understanding of ethanol deleterious effects on cardiac cells, however, is hindered by the clinical and experimental evidence that acute, moderate ethanol consumption exerts protective effects on the heart and the cardiovascular system (Lucas et al., 2005; Tolstrup, Nordestgaard, Rasmussen, Tybjærg-Hansen, & Grønbæk, 2008), in contrast to prolonged intake of high doses of ethanol, which promote the development of alcoholic cardiomyopathy (Dancy & Maxwell, 1986; Lucas et al., 2005; Tolstrup et al., 2008) and dilated cardiac hypertrophy (Dancy & Maxwell, 1986). Such a discrepancy has been explained with the dose of alcohol ingested and the way alcohol is metabolized within the cardiac myocyte. Due to the absence of alcohol dehydrogenase (EC 1.1.1.1), cardiac cells metabolize ethanol mainly through the alcohol-inducible cytochrome P450-2E1 (cyP4502E1, EC 1.14.13.n7) located within the sarcoplasmic reticulum (Tolstrup et al., 2008). Like alcohol dehydrogenase, cyP4502E1 converts ethanol to acetaldehyde, and it is to this metabolite that the deleterious effects of alcohol in cardiac muscle have been attributed (Zhang, Li, Brown, & Ren, 2004). In addition, oxidation of ethanol via cyP4502E1 is associated with the production of reactive oxygen species (ROS) and free radicals, and both these moieties contribute significantly to the development of alcoholic cardiac pathology (Tolstrup et al., 2008; Zhang et al., 2004).

In the present study, using a combination of animal and cellular models, we investigated the effects of acute and chronic exposure to ethanol on cardiac Mg^{2+} homeostasis. The reported results indicate that short-term exposure to low doses of ethanol does not result in Mg^{2+} extrusion or changes in cellular Mg^{2+} content, at variance with what is observed in liver cells. In contrast, prolonged and chronic exposure to high doses of ethanol elicits a major loss of Mg^{2+} from the cells. The modality of Mg^{2+} extrusion largely resembles that observed in hepatocytes in terms of time- and dose-dependence. The effect of ethanol is markedly reduced by inhibitors of cyP4502E1, suggesting that ethanol metabolism through the cytochrome is key to induce Mg^{2+} mobilization from cardiac cells. Consistent with this observation, a significant portion of cellular Mg^{2+} is lost from the sarcoplasmic reticulum where the cyP4502E1 is located. Overall, our data indicate that loss of cellular Mg^{2+} constitutes an essential component of the response of cardiac myocytes to ethanol exposure, as already observed in liver cells. Because of the close association of Mg^{2+} with ATP within cytoplasm and mitochondria (Günther, 1986; Romani & Scarpa, 1992; Scarpa & Brinley, 1981), and its role in regulating reticular Ca^{2+} cycling (Laver & Honen, 2008), it is tempting to speculate that Mg^{2+} loss may have significant repercussions on ATP utilization and contractility within the heart.

2. Materials and methods

2.1 Materials

Collagenase (CLS-I) was from Worthington Biochemical Corporation (Lakewood, NJ). All other chemicals were of analytical grade (Sigma-Aldrich, St. Louis, MO).

2.2 Animal 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.

2.3 Chronic alcohol model

Male Sprague-Dawley rats (180–200 g body weight) were randomly divided into control- and ethanol-treated groups and housed individually in metabolic cages. Ethanol-treated rats were maintained for 3 weeks on a 6% (v/v) ethanol Lieber-DeCarli diet. Pair-fed control rats received an isocaloric liquid control diet (Dyets, Bethlehem, PA), as previously reported (Torres, Konopnika, Berti-Mattera, Liedtke, & Romani, 2010; Young et al., 2003). Age-matched, Purina Chow pellet-fed control rats were used for comparison. Weight gain was recorded weekly. To study recovery, the ethanol diet was suspended after 3 weeks of alcohol administration, and rats of both experimental groups were fed the liquid control diet for a period of time varying from 2 to 15 days.

2.4 Determination of total Mg^{2+} and Na^+ content

Cardiac total Mg^{2+} , Na^+ , K^+ , and Ca^{2+} contents were measured in hearts of animals maintained on ethanol diet and liquid control diet as previously reported (Young et al., 2003). Briefly, the hearts were explanted, quickly rinsed in ice-cold sucrose (250 mM) solution, blotted on absorbing paper, and weighed. The atria were removed and the ventricles homogenized (10% w/v) in 250 mM sucrose using a Polytron homogenizer (15-sec pulses $\times$ 3). The homogenate was acidified by addition of HNO_3 (10% final concentration), and extracted overnight. Following sedimentation of denatured proteins (10,000 rpm $\times$ 3 min), the Na^+ and Mg^{2+} contents of the acid extract were measured by atomic absorbance spectrophotometry (AAS) in a Perkin-Elmer 3100 spectrometer calibrated with appropriate standards, and normalized per mg of protein and g of tissue.

2.5 Langendorff perfusion procedure

Following exposure to Lieber-DeCarli diet or to isocaloric control diet, the animals were anesthetized by intraperitoneal (i.p.) injection of a saturated pentobarbital solution (50 mg/kg body weight). Once deep anesthesia was attained (assessed by the disappearance of pain reflex and corneal touch), the chest was opened and the heart rapidly excised at the aortic arch. The aorta was mounted on a truncated 16-gauge needle and the heart was flushed with a medium containing (mM): NaCl, 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 connected to a perfusion pump and retrograde-perfused in a Langendorff 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 & Scarpa, 1990). 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-sec intervals, and the Mg^{2+} content measured by AAS. The first 10 min provided a baseline for the subsequent addition of the reported ethanol doses. Ethanol was diluted directly into the perfusion medium, and administered for 10 min (Fig. 1). 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

addition of ethanol 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 calculated taking into account the perfusion rate (7 mL/g/min) and the time of collection (30 sec), and expressed as mmol. 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 lactate dehydrogenase (LDH) activity in aliquots of the perfusate at 1-min intervals throughout the experimental procedure. The release of K^+ from potentially damaged cells was also measured by AAS in aliquots of the perfusate according to published protocol (Romani & Scarpa, 1990). 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.

2.6 Cardiac ventricular myocytes isolation

Cardiac ventricular myocytes were isolated by collagenase digestion as described previously (Romani et al., 1993; Romani & Scarpa, 1990). After isolation, myocytes were resuspended in a medium having the following composition (mM): NaCl, 120; KCl, 3; $CaCl_2$, 1.2; KH_2PO_4 , 1.2; glucose, 10; HEPES, 10; $NaHCO_3$, 12; pH 7.2 at 37 °C, containing 1.2 mM $MgCl_2$ (Romani et al., 1993; Romani & Scarpa, 1990). Cells were kept at room temperature, under constant flow of $O_2:CO_2$ 95:5 until used. Cell viability, assessed as LDH release and maintenance of rod shape, was found to be $82\% \pm 4\%$ ($n = 8$), and did not change significantly over the course of 4 h ($76\% \pm 5$, $n = 7$). In order to determine Mg^{2+} transport, 1 mL of myocytes suspension (2.5×10^5 cells) was transferred to a microcentrifuge tube, and the cells were rapidly sedimented at $600 \times g$ for 30 sec. The pellet was washed once with 1 mL of the Mg^{2+} -free medium described in the previous section. After the 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, varying concentrations of ethanol were added to the incubation system. At 2-min intervals, 0.7 mL of incubation mixture was withdrawn in duplicate, and the cells sedimented in microcentrifuge tubes. The supernatants were removed and Mg^{2+} content determined by AAS. The cell pellets were digested overnight in 10% HNO_3 . Following sedimentation of the denatured protein ($8,000 \times g$ for 5 min) in microcentrifuge tubes, Mg^{2+} and Na^+ content of the acid extract were measured by AAS.

In separate sets of experiments, cells were pre-treated with 4-methyl-pyrazole (4-MP, 50 μM) or chloromethiazole (CMZ, 100 μM) as inhibitors of cytochrome P4502E1 (Feierman & Cederbaum, 1985). Prior to ethanol addition, two aliquots of the medium (0.2 mL) were removed at 2-min intervals to establish extracellular Mg^{2+} baseline. Following ethanol addition, the incubation was continued for 90 additional minutes, withdrawing 0.2-mL aliquots of the medium at 15-min intervals. The medium aliquots were sedimented at 7,000 rpm $\times$ 1 min in microcentrifuge tubes to exclude possible artifacts due to cell lifting.

The supernatant was transferred to clean tubes and assessed for Mg^{2+} content by AAS. At the end of the experiment, any residual medium was removed by vacuum aspiration, and the

cells were digested in 0.5 mL 10% HNO₃ to measure residual cellular Mg²⁺ content by AAS as indicated above.

2.7 Cellular Mg²⁺ distribution

Total cellular Mg²⁺ content and distribution among cytoplasm, mitochondria, and other cellular organelles (e.g., sarcoplasmic reticulum and nucleus) were assessed. Cardiac ventricular myocytes were washed, and incubated in Mg²⁺-free medium as described above. 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 intervals, and aliquots of the incubation mixture were withdrawn and sedimented at 10,000 × g for 2 min. The 5-min interval between agent additions was used based upon preliminary observations indicating this lapse of time as optimal to mobilize Mg²⁺ from cytoplasm (digitonin), mitochondria (FCCP), and non-mitochondrial pools (A23187) (Fagan, Cefaratti, & Romani, 2004). FCCP was used to mobilize Mg²⁺ from mitochondria because its inhibitory effect on mitochondria promotes a marked Mg²⁺ efflux from the organelle (Akerman, 1981). The ionophore A23187 was used as the most common commercially available tool to mobilize Mg²⁺ from cellular pools (Fagan et al., 2004). The Mg²⁺ content in the supernatant was measured by AAS. Residual Mg²⁺ content in cell pellets was also measured by AAS after acid digestion performed as reported previously. The Mg²⁺ content present in the cell pellet and in the extracellular medium prior to the addition of any stimulatory agent were calculated and used as a baseline reference to determine the net amount of Mg²⁺ retained within the cell or released into the incubation medium, respectively.

2.8 Determination of ATP levels

Cardiac ATP levels were reported as described in Tessman and Romani, 1998. Briefly, following anesthesia of ethanol-exposed and control animals, the chest was opened and the heart excised. The atria were discarded and 0.25 g of cardiac ventricle muscle were removed, homogenized (20% wt/vol) in 5% perchloric acid, and digested for 10 min in ice. The acid mixture was neutralized by addition of 1 volume of 1 M KHCO₃, and the denatured protein was sedimented in a refrigerated Beckman J-6B centrifuge (1,500 × g for 10 min). The supernatants were removed and stored at -20 °C until used. Determination of ATP level was carried out by a luciferin-luciferase assay (detecting sensitivity in the pmol-nmol/mL range; Sigma-Aldrich) with a LUMAT Berthold LB 9501 luminometer or by high-pressure liquid chromatography (HPLC) with a C18 RP column (Millipore Waters) and 60 mM ammonium phosphate (pH 6.6 with ammonium hydroxide) as the mobile phase (Tessman & Romani, 1998).

Cellular ATP level was also measured in collagenase-dispersed cardiac ventricular myocytes. For this purpose, 700-µL aliquots of the cell incubation mixture were withdrawn, and the cells sedimented in microcentrifuge tubes (800 rpm for 2 min). The supernatants were removed, and the cells were digested in perchloric acid (5% final concentration) for 10 min in ice. The acid mixture was neutralized and processed as reported for the tissue homogenate. Adenine nucleotides standards (1–20 nmol/mL) were used for calibration of luminometer or HPLC.

2.9 Additional procedures

Aliquots of the perfusate were collected at 1-min intervals, and LDH activity was measured by an enzymatic kit (Sigma-Aldrich) sensitive to detect changes in the $\mu\text{U/mL}$ range, and expressed as U/L. Similar determinations were carried out in isolated cardiac myocytes and H9C2 cells. In both cell models, LDH activity was assessed as a percentage of the total amount of the enzyme released from digitonin-permeabilized cells.

Protein content was determined by Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951), using bovine serum albumin as a standard.

After excision of the heart, blood samples were collected by heparinized capillaries. The tubes were centrifuged at $500 \times g$ for 20 min, and plasma alcohol content was determined by enzymatic assay (Sigma-Aldrich). Exposure to Lieber-DeCarli diet for 3 weeks resulted in a plasma ethanol concentration of 42 ± 3 mmol/L ($n = 8$), which is consistent with other published reports (Fisher et al., 2001).

2.10 Statistical analysis

The data are reported as mean $\pm$ SE. Data were first analyzed by one-way ANOVA. Multiple means were then compared by Tukey's multiple comparison test performed with a q value established for statistical significance of $p < 0.05$.

3. Results

Exposure to ethanol in the diet for 3 weeks resulted in a significant loss of Mg^{2+} from cardiac tissue (Table 1). Consistent with a Mg^{2+} extrusion via the $\text{Na}^+/\text{Mg}^{2+}$ exchanger located in the sarcolemma of the cardiac ventricular myocytes (Cefaratti & Romani, 2007; Romani et al., 1993; Romani & Scarpa, 1990; Vormann & Günther, 1987), Mg^{2+} loss was associated with an increase in total Na^+ content (Table 1), and a decrease in K^+ content, while Ca^{2+} content trended toward an increase (Table 1). As observed in liver cells (Tessman & Romani, 1998), tissue ATP content also decreased following prolonged exposure to ethanol (Table 1). Removal of ethanol from the diet resulted in a slow but steady return of ATP and cation contents to basal conditions over a period of 10–12 days (Table 2).

Hearts of rats exposed to ethanol in the diet for 3 weeks were explanted and retrograde-perfused in a Langendorff system. In these hearts, acute ethanol administration did not induce a detectable Mg^{2+} extrusion, regardless of the dose of alcohol administered (Fig. 1A). In contrast, ethanol treatment of hearts from control animals that had received an isocaloric diet resulted in the extrusion of a significant amount of Mg^{2+} in the perfusate in a dose- and time-dependent manner (Fig. 1B). The net amount of Mg^{2+} extruded from the heart is reported in Fig. 1C. Doses higher than 0.5% ethanol (≈ 75 mM) were not used as they could result in an abnormal increase in membrane fluidity and release of cytosolic LDH.

The mechanism responsible for ethanol-induced Mg^{2+} extrusion from cardiac ventricular myocytes was further investigated in collagenase-dispersed cells. As Fig. 2 shows, collagenase-dispersed myocytes responded to alcohol administration by extruding Mg^{2+}

across the cell membrane in a dose- and time-dependent manner (Fig. 2A). The Mg^{2+} extrusion was observed as a net increase in the extracellular medium (Fig. 2A), or as a decrease in total cellular Mg^{2+} content (Fig. 2B). The extrusion of Mg^{2+} was associated with a decrease in K^+ content and an increase in Na^+ content. Cellular Ca^{2+} content also trended toward an increase (Table 3).

To determine whether ethanol elicited Mg^{2+} extrusion by depleting specific cellular magnesium compartments or had a more diffuse effect, cardiac myocytes were sequentially treated with digitonin, FCCP, and A23187 following 24 hours of exposure to different doses of ethanol. Because alternative techniques (e.g., electron probe X-rays microanalysis) are not available to us, we have developed this alternative approach to quantitate Mg^{2+} from the cytoplasm (digitonin), mitochondria (FCCP), and other, non-mitochondrial, cellular pools (A23187). This approach has been used successfully in various cell models (Fagan et al., 2004; Young, Berti-Mattera, & Romani, 2007). The results reported in Fig. 3 indicate that ethanol administration depleted all cellular Mg^{2+} pools in a dose-dependent manner.

Ethanol is oxidized to acetaldehyde through the cytosolic alcohol dehydrogenase (ADH, EC 1.1.1.1) and the reticular cytochrome P450 2E1 (CYP2E1, EC 1.14.13.n7). The latter process, however, is coupled with the production of reactive oxygen species, free radicals, and lipid peroxidation products within the cell (Tolstrup et al., 2008; Zhang et al., 2004). Cardiac ventricular myocytes lack alcohol dehydrogenase, and metabolize ethanol exclusively through CYP2E1. Experimental evidence indicates that inhibition of alcohol metabolism prevents ATP loss and Mg^{2+} extrusion in liver cells (Tessman & Romani, 1998).

Thus, we assessed the ability of 4-methylpyrazole (4-MP) and chloromethiazole (CMZ), which inhibit cyP4502E1 with different specificity, to prevent Mg^{2+} extrusion by blocking ethanol metabolism. Administration of 50 μM 4-MP inhibited Mg^{2+} extrusion by 1.1 ethanol by ~50% at the early time points (15 min and 30 min) and by ~30% at $t = 60$ min (Fig. 4A). Administration of 100 μM 4-MP did not provide a more effective protection (not shown), and higher concentrations of 4-MP were not tested. In contrast, administration of 100 μM CMZ inhibited by more than 50% the effect of 0.1% and 0.5% ethanol at all the time points (Fig. 4B). Administration of 150 μM CMZ inhibited Mg^{2+} extrusion by approximately 70%. No higher concentrations of CMZ were tested. Co-addition of 4-MP (50 μM) and CMZ (100 μM) did not attain higher inhibition than the inhibition observed with CMZ alone (not shown). The larger retention of cellular Mg^{2+} was associated with increased retention of cellular ATP content (Fig. 4C).

Consistent with what is observed in liver cells, pre-treatment with amiloride (500 μM , Fig. 5) or imipramine (200 μM , not shown) also prevented ethanol-induced Mg^{2+} extrusion to a significant extent.

In agreement with the data reported in Table 1, the loss of Mg^{2+} induced by ethanol was associated with a decrease in total ATP content (Fig. 6), although the decrease in phosphonucleotide content was significantly smaller (approximately 17%) than the

corresponding Mg^{2+} loss (approximately 25%). Administration of CMZ, 4-MP, or amiloride resulted in increased retention of cellular ATP content despite ethanol treatment (Table 4).

Discussion

Consumption of small doses of ethanol is thought to have beneficial cardiovascular effects (Lucas et al., 2005). In contrast, prolonged consumption of alcohol, especially in high doses, results in the development of alcoholic cardiomyopathy in human subjects (Brown, Crawford, Natavio, Petrovski, & Ren, 1998; Lucas et al., 2005). The disease has been attributed to the oxidation of ethanol to acetaldehyde by the cyP4502E1 located within the sarcoplasmic reticulum of the cardiac myocyte (Zhang et al., 2004). Not only is acetaldehyde toxic *per se* due to its ready reactivity with phospholipids and proteins (Zhang et al., 2004), but its metabolic conversion by cyP4502E1 is coupled with the production of reactive oxygen species, free radicals, and lipid peroxidation products, which also react with and damage proteins, enzymes, and other membrane components (Lucas et al., 2005). As cyP4502E1 is substrate-induced, any increase in its operation results in the enhanced production of deleterious by-products and cellular damage. Thus, the activation of cyP4502E1 can reasonably explain the damaging effects of chronic ethanol consumption on cardiac functions. The production of large quantities of acetaldehyde, reactive oxygen species, and lipid peroxidation products within cardiac myocytes depresses the cells' contractile machinery and function (Oba, Maeno, Nagao, Sakuma, & Murayama, 2008), and promotes the release of detectable amounts of troponin C into the extracellular space (Vary & Deiter, 2005). The final outcome of this series of events is a decrease in cardiac contractility, which leads to the development of alcoholic cardiomyopathy.

While attention has been paid to the effect of acute and chronic ethanol administration on cellular and reticular Ca^{2+} homeostasis due to its role in myocyte contractility, no information is currently available about the effects of ethanol administration and metabolism on cardiac Mg^{2+} homeostasis. Magnesium is abundantly present within the main cellular compartments of the cardiac myocyte (Romani & Scarpa, 1992; Wolf et al., 2003), and evidence in the literature indicates that changes in cellular and extracellular Mg^{2+} play major roles in cardiac physiology. Physiological levels of extracellular Mg^{2+} have been associated with proper action potential duration and regulation of Na^+ and Ca^{2+} channels (Howarth, Waring, Hustler, & Singh, 1994). In agreement with this observation, an increased risk of ischemic heart disease (Swaminathan, 2003) and several cardiac-related conditions, including specific forms of arrhythmias and long QT syndrome, have been associated with a less than optimal Mg^{2+} content within the cardiac tissue (Martin, González, & Slatopolsky, 2009).

Moreover, Mg^{2+} supplementation has been reported to ameliorate the myocardial dysfunction associated with alcoholic cardiomyopathy, renormalizing heart size, isometric force, and isotonic shortening (Brown et al., 1998). How exactly Mg^{2+} elicits these effects has not been fully investigated. Because Mg^{2+} acts as a natural Ca^{2+} -channel blocker, it is possible that changes in Ca^{2+} -mediated force development and cardiac myocyte shortening depend on the restoration and maintenance of physiological $[Ca^{2+}]_i$ and whole cell Ca^{2+} homeostasis, with direct implications for contractile myofilaments' functions. In addition,

restoration of normal cellular Ca^{2+} levels would also limit the progression of cardiac hypertrophy by attenuating Ca^{2+} -mediated signaling leading to hypertrophy (Molkentin, 2004). Whether Mg^{2+} may also play a direct role in these events by regulating protein synthesis and mRNA translation (Maguire & Cowan, 2002) is undefined.

In the present study, we investigate the effect of exposure to ethanol on Mg^{2+} homeostasis in cardiac myocytes. The reported results suggest that ethanol metabolism via cytP4502E1 promotes Mg^{2+} loss predominantly from cytoplasm, mitochondria, and sarcoplasmic reticulum. Evidence for the occurrence of such a loss is that 1) total cellular and subcellular Mg^{2+} decrease in a time-dependent manner that directly correlates to the amount of ethanol administered, and 2) inhibition of alcohol metabolism by CMZ or 4-MP prevents the loss of Mg^{2+} from cardiac cells. It is worth nothing that acute administration of ethanol to hearts of animals exposed to an elevated concentration of ethanol through the Lieber-DeCarli diet does not elicit Mg^{2+} extrusion into the perfusate, regardless of the dose of ethanol administered acutely (Fig. 1A). As these hearts already presented a marked Mg^{2+} loss (Table 1) prior to the acute perfusion with ethanol (Fig. 1A), the suggestion is that cardiac cells have a limited pool of mobilizable Mg^{2+} . Depletion of this pool by the chronic exposure to ethanol renders the cardiac myocytes unresponsive to subsequent acute addition of ethanol. Alternatively, or in addition, it is possible that the lack of response to acute ethanol addition depends on the down-regulation of cytP4502E1 exerted by the prolonged exposure to ethanol through the diet. Discrimination between these two possibilities will be the object of future studies.

Cardiac cells extrude Mg^{2+} physiologically across the sarcolemma through a $\text{Na}^+/\text{Mg}^{2+}$ exchanger (Cefaratti & Romani, 2007; Romani et al., 1993). Presently, no specific inhibitor of the exchanger is commercially available. However, amiloride, imipramine, and quinidine have been widely used as non-specific inhibitors of the transporter (reviewed in Romani & Scarpa, 1992, and Wolf et al., 2003). Consistent with these premises and with data obtained from liver cells (Tessman & Romani, 1998), administration of amiloride or imipramine prevents ethanol-induced Mg^{2+} extrusion from cardiac myocytes. These results would be consistent with the notion that Mg^{2+} is extruded in exchange for extracellular Na^+ , which does indeed increase under our experimental conditions (Table 1 and Table 3), rather than exit the cell due to a non-specific, ethanol-induced increase in sarcolemma permeability. How the $\text{Na}^+/\text{Mg}^{2+}$ exchanger is activated following ethanol exposure is presently undetermined. As limited information is available about the Mg^{2+} sensitivity of the transporter, it cannot be excluded that it senses the increase in free Mg^{2+} resulting from the decrease in ATP buffering within the cytoplasm (data reported here and in Tessman & Romani, 1998). Along these lines, retention of cellular Mg^{2+} is associated with more sustained ATP levels within the cardiac myocyte. While higher levels of ATP can help explain Mg^{2+} retention in cytoplasm and mitochondria through its buffering capability, it is less clear why retention of Mg^{2+} by amiloride pre-treatment in the presence of ethanol results in higher cellular ATP levels than those observed in the absence of the $\text{Na}^+/\text{Mg}^{2+}$ exchanger inhibitor. This result would indicate that the decrease in ATP levels does not simply depend on ethanol metabolism, and suggests the intriguing possibility that retention of Mg^{2+} within cytoplasm and perhaps mitochondria either has protective effects on ATP hydrolysis or promotes a more sustained ATP synthesis in the mitochondria. Cytoplasm,

mitochondria, and sarcoplasmic reticulum constitute the three main Mg^{2+} compartments within cardiac myocytes (Günther, 1986; Romani & Scarpa, 1992; Wolf et al., 2003), and Mg^{2+} plays a significant role in each of them by controlling ATP production and utilization, cardiac bioenergetics, and Ca^{2+} release and cycling, respectively (Romani & Scarpa, 1992; Wolf et al., 2003). Thus, loss of Mg^{2+} from these compartments can affect various bioenergetics and metabolic processes to a varying extent. Adenosine triphosphate is the main agent forming a complex with Mg^{2+} within cytoplasm and mitochondria. Hence, loss of ATP as a result of ethanol metabolism and/or imbalance between ATP synthesis and utilization limits the ability of cardiac myocytes to retain significant amounts of Mg^{2+} within cytoplasm and mitochondria until cellular ATP levels are restored to a sufficient extent (Table 2). Mitochondria depend on a proper Mg^{2+} homeostasis and Ca^{2+}/Mg^{2+} ratio to maintain an optimal ATP level within cardiac myocytes. A decrease in matrix Mg^{2+} level has been associated with structural and functional alteration of mitochondrial complexes and dehydrogenases (Panov & Scarpa, 1996), and with a marked decrease in mitochondrial respiratory rate (Panov & Scarpa, 1996). A less than optimal ATP level within the cardiac myocyte will affect the ability of the cell to maintain proper basal (diastolic) Ca^{2+} level in the cytoplasm, and to maintain the trans-membrane Na^{+} and K^{+} gradients necessary for effective cardiac action potential and contractility (Howarth et al., 1994).

It is worth noting that removal of ethanol from the incubation system does not result in a rapid restoration of Mg^{2+} level. Cardiac cells from animals exposed to alcohol for 3 weeks require 10 days or so to restore their cellular Mg^{2+} levels to pre-ethanol levels (Table 2). A similar time course has been observed in liver cells (Torres et al., 2009) and is related to the defective translocation of PKC ϵ from cytoplasm to cell membrane (Torres et al., 2010).

Because PKC regulates Mg^{2+} accumulation also in cardiac myocytes (Romani, Marfella, & Scarpa, 1992), it is possible that defects in PKC translocation also occur in cardiac myocytes upon exposure to alcohol. The return of cellular Mg^{2+} to physiological levels correlates with respect to time with the restoration of cellular ATP content. Thus, it is likely that restoration of cellular ATP content plays a major role in favoring retention of Mg^{2+} within the cytoplasm and the mitochondrial matrix, and in re-establishing proper cellular Mg^{2+} content. While the presence of Mrs2 in the mitochondrial membrane provides a suitable entry channel to restore mitochondrial Mg^{2+} level (Kolisek et al., 2003), it is less clear which mechanism or mechanisms are responsible for restoring Mg^{2+} content within the sarcoplasmic reticulum of the cardiac myocyte. Moreover, we have been unable to detect how cytosolic $[Mg^{2+}]_i$ changes following ethanol administration and oxidation due to concomitant changes in fluorescence of endogenous pyridine nucleotides, and opposite changes in Ca^{2+} level (reviewed in Romani, 2008), which all interfere with fluorimetric determinations of $[Mg^{2+}]_i$. As new fluorescent indicators applicable to single cell determination and intracellular magnesium quantifications become available (Malucelli et al., 2014), these important aspects of cellular Mg^{2+} homeostasis will be better characterized and understood without limitations associated with current laborious manipulations.

Overall, Mg^{2+} extrusion appears to be part of the cellular response to ethanol exposure. It is presently unclear, however, to which extent Mg^{2+} loss is involved in the onset of the pathological changes observed in alcoholic cardiomyopathy, as changes in other cations

such as Ca^{2+} and Na^{+} certainly play a more important and better codified role in the onset of the pathology, including activation of Ca^{2+} -dependent signaling mechanism and changes in membrane potential. Further studies are necessary to understand the possible contribution of Mg^{2+} loss to this scenario.

To our knowledge, this study is the first to investigate changes in Mg^{2+} homeostasis in cardiac myocytes following acute and chronic exposure to ethanol. The results provided by this study provide much needed background information and unveil some interesting lines of research to investigate in more detail the consequence of ethanol-induced Mg^{2+} loss for cardiac cell bioenergetics and contractile function.

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HIGHLIGHTS

1. Chronic exposure to Ethanol markedly depleted Cardiac myocytes of cellular magnesium.
2. Acute ethanol exposure induced a time- and dose-dependent manner Mg^{2+} loss from cardiac cells.
3. Inhibition of cytP4502E1 prevents ethanol effect on cellular magnesium
4. Magnesium extrusion across the sarcolemma occurs via the amiloride-inhibited Na^+/Mg^{2+} exchanger.

Figure 1. Ethanol-induced Mg^{2+} extrusion in perfused hearts

Hearts from rats fed a Lieber-DeCarli diet for 3 weeks (Fig. 1A) or an isocaloric control diet (Fig. 1B) were perfused in a Langendorff system as reported in Materials and methods. At the time reported in the figures, different doses of ethanol were administered for 10 min. The net amount of Mg^{2+} extruded by the hearts is reported in Fig. 1C. Data are means $\pm$ S.E. of four different hearts per experimental condition. In Fig. 1B, all the data points under the curve of extrusion were statistically significant vs. the control points. Labeling is omitted for simplicity. Fig. 1C: *statistically significant vs. ethanol-untreated hearts; #statistically significant vs. 0.1%-treated samples.

Figure 2. Ethanol-induced Mg^{2+} extrusion in collagenase-dispersed cardiac ventricular myocytes Cardiac ventricular myocytes were isolated by collagenase digestion and incubated with different doses of ethanol as reported in Materials and methods. Fig. 2A shows a typical Mg^{2+} extrusion profile. Net Mg^{2+} extrusion is shown in Fig. 2B. Data are means $\pm$ S.E. of five different preparations, each tested in duplicate. *Statistically significant vs. control values; #statistically significant vs. 0.1%-treated samples.

Figure 3. Cellular Mg^{2+} distribution following ethanol-stimulation

Collagenase-dispersed cardiac ventricular myocytes were incubated in the presence of varying ethanol concentrations. At the end of the incubation, cells were gently sedimented, resuspended, and treated sequentially with digitonin, FCCP, and A23187 (Materials and methods). The figure inset indicates the time of addition of each of these agents and the changes in extracellular Mg^{2+} content. The total cellular Mg^{2+} amount prior to the addition of digitonin, and the estimated Mg^{2+} content mobilized from cytoplasm (digitonin), mitochondria (FCCP), and non-mitochondrial compartments (A23187) are reported in the main figure. A typical experiment is shown in the figure inset. Data reported in main Fig. 3 are mean $\pm$ S.E. of five different cell isolations, each tested in duplicate for all the experimental conditions reported. *Statistically significant vs. control value.

Figure 4. Ethanol-induced Mg²⁺ extrusion in collagenase-dispersed cardiac ventricular myocytes pretreated with cyp4502e1 inhibitors

Cardiac ventricular myocytes isolated by collagenase digestion were incubated in the presence of different doses of ethanol, in the absence and in the presence of 100 μ M CMZ (Fig. 4A) or 50 μ M 4MP (Fig. 4B). Data are means $\pm$ S.E. of five different preparations, each tested in duplicate. *Statistically significant vs. ethanol-only treated sample.

Figure 5. Ethanol-induced Mg^{2+} extrusion in collagenase-dispersed cardiac ventricular myocytes pretreated with amiloride

Cardiac ventricular myocytes isolated by collagenase digestion were incubated in the presence of different doses of ethanol, in the absence and in the presence of 500- μ M amiloride. Data are means $\pm$ S.E. of five different preparations, each tested in duplicate. All the data points of ethanol plus amiloride-treated cells are statistically significant vs. ethanol-only treated samples. Labeling is omitted for simplicity.

Table 1

Cation and ATP contents in hearts of rats fed for 3 weeks a Lieber-DeCarli diet or an isocaloric control liquid diet

	Mg²⁺	Na⁺	K⁺	Ca²⁺	ATP
Control Diet	69.3 ± 4.1	310.7 ± 17.2	987.4 ± 33.8	9.2 ± 1.4	4.76 ± 0.47
Ethanol Diet	58.4 ± 3.0 [#]	374.6 ± 13.5 [#]	873.5 ± 25.2 [#]	12.0 ± 0.9	3.27 ± 0.23 [#]
Significance	<i>p</i> < 0.046	<i>p</i> < 0.001	<i>p</i> < 0.015	ns	<i>p</i> < 0.011

Cardiac cation and ATP contents are reported as nmol/mg protein (means ± SE, *n* = 10 for each experimental group).

[#] Statistically significant versus corresponding control value.

Table 2

Cation and ATP contents in hearts of ethanol-fed rats fed at different times after removal of ethanol from the diet

	Mg²⁺	Na⁺	K⁺	Ca²⁺	ATP
2 days	60.8 ± 5.1	362.3 ± 21.3	890.8 ± 31.2	11.4 ± 1.2	3.58 ± 0.41
5 days	62.4 ± 3.5	348.6 ± 23.2	918.6 ± 28.6	10.7 ± 0.8	3.99 ± 0.73
8 days	64.8 ± 4.0	328.3 ± 26.8	947.2 ± 40.2	9.6 ± 1.7	4.29 ± 0.38
12 days	68.7 ± 6.3	303.5 ± 18.9	965.0 ± 35.6	9.1 ± 1.0	4.47 ± 0.54
15 days	70.8 ± 6.2	300.2 ± 25.4	971.8 ± 30.3	8.9 ± 0.9	4.58 ± 0.77

Cardiac cation and ATP contents are reported as nmol/mg protein (means ± SE, *n* = 6 for each experimental group).

Table 3

Cation contents in cardiac ventricular myocytes exposed for 60 min to 0.1% or 0.5% ethanol

	Mg ²⁺	Na ⁺	K ⁺	Ca ²⁺
Control	48.31 ± 0.20	40.47 ± 0.23	203.22 ± 0.49	2.89 ± 0.061
0.1% ethanol	44.60 ± 0.27 *	44.93 ± 0.29 *	190.08 ± 0.63 *	3.51 ± 0.079 *
0.5% ethanol	42.34 ± 0.32 *	48.39 ± 0.23 *	181.25 ± 0.56 *	4.03 ± 0.084 *

Cardiac cation contents are reported as nmol/mg protein (means ± SE, *n* = 4 for each experimental group).

* Statistically significant versus corresponding control value

Table 4

ATP and cation contents in cardiac ventricular myocytes exposed for 60 min to 0.1% or 0.5% ethanol

	ATP	Na ⁺	K ⁺	Ca ²⁺
Control	4.56 ± 0.091	39.89 ± 0.32	202.87 ± 0.57	2.91 ± 0.052
Control + CMZ	4.65 ± 0.082	40.14 ± 0.27	201.24 ± 0.58	2.89 ± 0.060
Control + 4-MP	4.67 ± 0.054	40.31 ± 0.35	200.75 ± 0.65	2.93 ± 0.053
Control + Ami	4.63 ± 0.045	40.15 ± 0.25	203.15 ± 0.49	2.90 ± 0.057
0.1% ethanol	3.79 ± 0.102 [*]	45.09 ± 0.27 [*]	188.76 ± 0.55 [*]	3.52 ± 0.055 [*]
0.1% ethanol + CMZ	4.31 ± 0.086 [#]	42.45 ± 0.30 [#]	194.56 ± 0.62 [#]	3.20 ± 0.064 [#]
0.1% ethanol + 4-MP	4.25 ± 0.091 [#]	43.56 ± 0.35 [#]	192.39 ± 0.70 [#]	3.27 ± 0.079 [#]
0.1% ethanol + Ami	4.31 ± 0.074 [#]	41.98 ± 0.28 [#]	197.71 ± 0.52 [#]	3.12 ± 0.070 [#]
0.5% ethanol	3.38 ± 0.114 [*]	48.17 ± 0.28 [*]	179.86 ± 0.63 [*]	3.98 ± 0.069 [*]
0.5% ethanol + CMZ	4.11 ± 0.102 [#]	43.76 ± 0.39 [#]	188.31 ± 0.59 [#]	3.21 ± 0.076 [#]
0.5% ethanol + 4-MP	4.12 ± 0.104 [#]	44.38 ± 0.32 [#]	184.50 ± 0.68 [#]	3.32 ± 0.087 [#]
0.5% ethanol + Ami	4.11 ± 0.083 [#]	42.87 ± 0.45 [#]	192.34 ± 0.72 [#]	3.13 ± 0.082 [#]

Cardiac ventricular myocytes isolated by collagenase digestion were incubated in the presence of different doses of ethanol, both in the absence and in the presence of 100-μM CMZ, 50-μM 4-MP, or 500-μM amiloride. Data are reported as nmol/mg protein, and are means ± S.E. of four different preparations, each tested in duplicate.

* Statistically significant vs. control value.

[#] Statistically significant vs. ethanol-only treated sample.