Increased [Mg\textsuperscript{2+}]\textsubscript{o} reduces Ca\textsuperscript{2+} influx and disruption of mitochondrial membrane potential during reoxygenation

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Sharikabad, Mohammad Nouri, Kirsten Margrethe Østbye, and Odd Brørs. Increased [Mg\textsuperscript{2+}]\textsubscript{o}, reduces Ca\textsuperscript{2+} influx and disruption of mitochondrial membrane potential during reoxygenation. Am J Physiol Heart Circ Physiol 281: H2113–H2123, 2001.—Increase in extracellular Mg\textsuperscript{2+} concentration ([Mg\textsuperscript{2+}]\textsubscript{o}) reduces Ca\textsuperscript{2+} accumulation during reoxygenation of hypoxic cardiomyocytes and exerts protective effects. The aims of the present study were to investigate the effect of increased [Mg\textsuperscript{2+}]\textsubscript{o} on Ca\textsuperscript{2+} influx and efflux, free cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) and Mg\textsuperscript{2+} concentrations ([Mg\textsuperscript{2+}]\textsubscript{i}); Ca\textsuperscript{2+} accumulation in the presence of inhibitors of mitochondrial or sarcoplasmatic reticulum Ca\textsuperscript{2+} transport, and finally mitochondrial membrane potential (\Delta\phi\textsubscript{m}). Isolated adult rat cardiomyocytes were exposed to 1 h of hypoxia and subsequent reoxygenation. Cell Ca\textsuperscript{2+} was determined by 45Ca\textsuperscript{2+} uptake, and the levels of [Mg\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{i} were determined by flow cytometry as the fluorescence of magnesium green and fluo 3, respectively. Ca\textsuperscript{2+} influx rate was significantly reduced by \sim 40%, whereas Ca\textsuperscript{2+} efflux was not affected by increased [Mg\textsuperscript{2+}]\textsubscript{o}. Clonazepam, a selective mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange inhibitor (100 \mu M), significantly reduced Ca\textsuperscript{2+} accumulation by 70% and in combination with increased [Mg\textsuperscript{2+}]\textsubscript{o} by 90%. Increased [Mg\textsuperscript{2+}]\textsubscript{o} also preserves \Delta\phi\textsubscript{m} when added with the cationic dye JC-1 by flow cytometry. A significant inverse correlation was observed between \Delta\phi\textsubscript{m} and cell Ca\textsuperscript{2+} in reoxygenated cells treated with increased [Mg\textsuperscript{2+}]\textsubscript{o}, and clonazepam. In conclusion, increased [Mg\textsuperscript{2+}]\textsubscript{o} (5 mM) inhibits Ca\textsuperscript{2+} accumulation by reducing Ca\textsuperscript{2+} influx and preserves \Delta\phi\textsubscript{m}, without affecting [Ca\textsuperscript{2+}]\textsubscript{i}, during reoxygenation. Preservation of mitochondria may be an important effect whereby increased [Mg\textsuperscript{2+}]\textsubscript{o} protects the postischemic heart.

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locus (loci) of action. The latter was also the reason for investigating the effect of increased [Mg2+]i on Δψm during reoxygenation.

MATERIALS AND METHODS

Chemicals and materials. The following chemicals were obtained from Sigma (St. Louis, MO): BSA (essentially fatty acid-free), DL-carnitine, dibutyl phthalate, ouabain, trypsin, cyclopiacionic acid, calciumcin, and SDS. Joklik’s minimum essential medium (MEM) was obtained from Life Technologies (Paisley, UK). Collagenase and deoxyribonuclease were obtained from Worthington Biochemical (Lakevok, NJ). NPB-1 contained NPB supplemented with CaCl2, 0.8 mM MgSO4, and 0.1% (wt/vol) BSA. Aliquots of NPB were composed of (in mM) 120.0 NaCl, 3.3 KCl, 1.2 KH2PO4, 5.5 mM glucose. In hypoxia, cells were added to NPB-2 (no glucose added) equilibrated with 95% N2-5% CO2 (P02 = 1 kPa). At reoxygenation, gas was changed to 95% air-5% CO2, and buffer was supplied with 5.5 mM glucose. Oxygen tension in the test flasks was checked using an oxygen electrode (WTW Microprocessor Oximeter Oxi 96, Wissenschaftliche Technische Werkstätten; Weilheim, Germany) near the bottom of the Erlenmeyer flask where cells were added. Each Erlenmeyer flask was supplied with an inlet and outlet for gas and continuously gassed for the indicated time.

Determination of cell Ca2+ and influx kinetics of Ca2+. Cell Ca2+ was determined as rapidly exchangeable Ca2+ by uptake of 45Ca2+ and calculated from the 45Ca2+ content of the cells and the known specific activity of 45Ca2+ in the uptake buffer. Cell membrane Ca2+ transport was stopped by pipetting samples into centrifuge tubes with ice-cold buffer layered over an oil mixture, and cells were separated from buffer by subsequent centrifugation as previously described (34). The data were normalized to the protein content in each cell pellet. The Ca2+ influx rate was determined from the initial, apparently linear uptake of 45Ca2+ after the addition of isotope and exchange of 45Ca2+ from the apparent equilibrium of 45Ca2+ uptake (plateau level).

Determination of efflux kinetics of Ca2+. Decline of 45Ca2+ content in cells exposed to hypoxia and reoxygenation was used to evaluate Ca2+ efflux. Briefly, cells were loaded with trace amounts of 45Ca2+ during 1 h of hypoxia and 0.5 h of reoxygenation. The cell suspension was then centrifuged (250 g for 30 s), the supernatant was aspirated, and the cells were resuspended in oxygenated buffer (37°C) to dilute extracellular 45Ca2+ and start efflux of the isotope. After 5 min, the centrifugation and resuspension steps were repeated. For each dilution step, the extracellular isotope was diluted by a factor of ~10. To characterize the kinetics of the decline, the radioactivity remaining in the cells was normalized to protein content and subtracted the calculated equilibrium value (the prewash value divided by the product of both dilution factors).

Flow cytometric analysis of [Ca2+]i and [Mg2+]i. Levels of [Ca2+]i and [Mg2+]i were measured by flow cytometry as the fluorescence of fluo 3 and MgG, respectively. Fluo 3-AM and MgG are membrane permeable. After entering the cells, these esters are cleaved by intracellular esterases to yield the relatively cell-impermeant fluorescent indicators fluo 3 and MgG respectively. Fluo 3-AM and MgG were dissolved in DMSO right before each experiment, added to the incubation buffer at the time of addition (10 μM DMSO and 0.04% PEG). Clonazepam was dissolved in DMSO (100 mM) and diluted further to 1:1,000 in the incubation buffer at the start of experiments (100 μM clonazepam and 0.1% DMSO). In the experiments in which clonazepam was tested, 0.1% DMSO was added to all test conditions to exclude any effects of DMSO alone. The stock solution of 6-Carbamoyl-1-maleimide (MgG) was made in PBS (0.1 M, pH 7.4) and further diluted to 1:1,000 in the incubation buffer at the time of addition (10 μM DMSO and 0.04% PEG). Flow cytometric analysis of [Ca2+]i and [Mg2+]i. Levels of [Ca2+]i and [Mg2+]i were measured by flow cytometry as the fluorescence of fluo 3 and MgG, respectively. Fluo 3-AM and MgG are membrane permeable. After entering the cells, these esters are cleaved by intracellular esterases to yield the relatively cell-impermeant fluorescent indicators fluo 3 and MgG, respectively. Fluo 3-AM and MgG were dissolved in DMSO right before each experiment, added to the incubation buffer (NPB-2) to give the final concentration of 4.5 μM fluo 3 or 5 μM MgG (0.5% DMSO), and were present during the whole experiment. Samples were directly applied to the flow cytometer without any washing step to remove the extracellular indicators. Preliminary testing showed that the level of fluorescence in all groups (normoxic control, hypoxia/reoxygenation ± Mg2+) was slightly reduced, and to the same degree by a washing step, probably due to some loss of probe from the cells. A FACSort (Becton-Dickinson; Rutherford, NJ) flow cytometer equipped with a 488-nm argon ion laser and supplied with the Cell Quest software was used to measure fluorescence signals of fluo 3 and MgG in the cells. Fluo 3 and MgG, unlike ultraviolet light-excited indicators (e.g., fura 2), do not exert any spectral shift. Signals were obtained using a 530-nm bandpass filter (FL-1 channel) for both indicators. Each determination was based on a mean fluorescence intensity of 5,000 cells in arbitrary units. The fluorescence intensity of unloaded cells (background signal) of both indicators was ~2% of signals of indicator-loaded cells.

Calibration of fluo 3 and MgG fluorescence. Cells were loaded with the indicator for 1 h in NPB-2 (normoxic, 37°C
and 5.5 mM glucose). The cell suspension was then centrifuged (250 g for 30 s). The cell pellet was dispersed in 25-ml NPB-2 (nominally Ca²⁺ and Mg²⁺ free) and centrifuged (250 g for 30 s). The cell pellet was then washed twice with 5 ml of zero-Ca²⁺ buffer (calcium calibration buffer kit No. 2, Molecular Probes) in the case of fluo 3-loaded cells and Mg²⁺ calibration buffer component A (see MATERIALS AND METHODS) for MgG-loaded cells. Fluo 3-loaded cardiomyocytes were then exposed to various Ca²⁺ concentrations (0, 58, 135, 315, and 1,215 nM). [Ca²⁺] was equilibrated with extracellular Ca²⁺ by addition of the ionophore calcimycin (50 μM). MgG-loaded cells were exposed to various Mg²⁺ concentrations (0, 0.18, 0.36, 0.72, 1.44, 2.88, and 5.76 mM). [Mg²⁺] was equilibrated with extracellular Mg²⁺ by the addition of calcimycin (50 μM). Different Mg²⁺ concentrations were made by mixing Mg²⁺ calibration buffer components A and B [component A was composed of (in mM) 115 KCl, 20 NaCl, and 10 Tris (pH 7)] and component B was composed of (in mM) 35 MgCl₂, 115 KCl, 20 NaCl, and 10 Tris (pH 7.05)]. These were made using the recipe of the Mg²⁺ calibration standard kit (M-3120), which is no longer produced by Molecular Probes. Each determination was based on a mean fluorescence intensity of 5,000 cells in arbitrary units. The particular indicator (4.5 μM flou 3 or 5 μM MgG) was present in all solutions used in the signal calibration protocol in likeness with the experiments. Ca²⁺ binding [dissociation constant (Kd) of 325 nM] results in an increase in fluorescence intensity of flou 3 by ~100-fold. Flou 3 has an eight times higher affinity for Ca²⁺ than Mg²⁺ (data given by Molecular Probes). However, MgG, like other tricarboxylate aminophenol tria cetic acid chelators, binds Ca²⁺ with high affinity. The interference with Mg²⁺ measurements due to Ca²⁺ binding becomes significant when Ca²⁺ concentrations exceed ~1 μM (13, 18). Leysens et al. (22) found that the Kd of MgG for Ca²⁺ was 4.7 μM in rat cardiomyocytes, whereas the Kd of MgG for Mg²⁺ is ~1 mM. Consequently, we did exactly the same calibration experiments mentioned above but incubated instead the MgG-loaded cells with Ca²⁺ calibration buffers and flou 3-loaded cells with Mg²⁺ calibration buffers.

Δψm measurements with JC-1. The dye JC-1 has recently been evaluated as an optimal dye for measuring Δψm in cardiomyocytes (24). JC-1 is a lipophilic and cationic dye that exhibits potential-dependent accumulation in negatively charged mitochondria. At low concentrations (low Δψm), JC-1 exists primarily in a monomeric form, which emits green fluorescence. JC-1 at high concentrations (high Δψm) forms aggregates called “J” complexes, which emit red fluorescence at 590 nm. Thus a reduction in the ratio of red to green fluorescence indicates a fall in Δψm. Cells were loaded with 12 μM JC-1 for 10 min at 37°C and then immediately applied to the flow cytometer for signal recording using the 530-nm (FL-1 channel) and 585-nm (FL-2 channel) bandpass filters simultaneously. Each determination was based on the ratio of red to green mean fluorescence intensities measured in arbitrary units from 5,000 cells.

Statistics. All experiments were performed as paired comparisons. Statistical analysis was performed using Statgraphics Plus software (version 4.0, Manugistics; Rockville, MD). Statistical analysis of multigroup comparisons was performed by one-way ANOVA, and the method to discriminate among the means was Fisher’s least significant difference (LSD) method. Statistical analysis of two-sample comparisons was performed by Student’s t-test (two-sided). P < 0.05 was considered to be significant.

RESULTS

Cell Ca²⁺ during normoxia and reoxygenation of hypoxic cardiomyocytes. Cell Ca²⁺ was in the range of 2.5–3 nmol/mg protein at apparent equilibrium in the normoxic cells. ⁴⁴Ca²⁺ uptake reached a plateau after 1–2 min irrespective of adding ⁴⁴Ca²⁺ to the buffer at 10 or 120 min and was virtually unchanged during the time course of the experiments (Fig. 1). In cells exposed to hypoxia and reoxygenation, cell Ca²⁺ significantly increased after reoxygenation from 3 to 12 nmol/mg protein (Fig. 1). The equilibrium values for exchangeable cell Ca²⁺ in reoxygenated cells at 180 min were similar irrespective of adding ⁴⁴Ca²⁺ at the start of incubation or at 120 min (Fig. 1). ⁴⁴Ca²⁺ apparently equilibrated more slowly with the pool of exchangeable Ca²⁺ in reoxygenated cells than in normoxic cells at 120 min (Fig. 1, inset).

Effect of increased [Mg²⁺] on cell Ca²⁺ during hypoxia and reoxygenation. To investigate whether the effect of Mg²⁺ was exerted during hypoxia or reoxygenation, [Mg²⁺] was increased to 5 mM either from the start of the experiments or

![Fig. 1. Cell Ca²⁺ during normoxia and reoxygenation of hypoxic cardiomyocytes. ⁴⁴Ca²⁺ was added 10 min after start of experiments to the incubation buffer of normoxic control cells (●) or added from start to cells exposed to 1 h of hypoxia and 2 h of reoxygenation (○). Alternatively, ⁴⁴Ca²⁺ was added after 2 h to control cells (●) and cells exposed to 1 h of hypoxia and 1 h of reoxygenation (X). Each point represents the mean ± SE of 4–8 determinations from 2 different cell isolations. Inset: plot on a log vertical scale of the uptake kinetics of Ca²⁺ in control cells (●) and hypoxia-reoxygenation (H/R)-treated cells (X) when ⁴⁴Ca²⁺ was added at 2 h, expressed as the percent remaining to be taken up as a function of time.](http://ajpheart.physiology.org/)

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at the onset of reoxygenation. Both treatments reduced reoxygenation-mediated Ca\(^{2+}\) accumulation to the same degree (50%) compared with hypoxia- and reoxygenation-treated cells in the presence of normal [Mg\(^{2+}\)]\(_{o}\) (0.8 mM).

Lowering [Mg\(^{2+}\)]\(_{o}\) to 0.3 mM during hypoxia-reoxygenation did not alter cell Ca\(^{2+}\) accumulation compared with normal [Mg\(^{2+}\)]\(_{o}\) (0.8 mM) (Fig. 2).

Effect of increased [Mg\(^{2+}\)]\(_{o}\) on Ca\(^{2+}\) influx during reoxygenation. Trace amounts of 45Ca\(^{2+}\) were added to cells exposed to 60 min of hypoxia and 30 min of reoxygenation. [Mg\(^{2+}\)]\(_{o}\) increased to 5 mM at the onset of reoxygenation significantly reduced the initial rate of 45Ca\(^{2+}\) uptake (up to 5 min), reflecting a reduced influx velocity of Ca\(^{2+}\) (Fig. 3A). Because 45Ca\(^{2+}\) uptake was apparently not linear for 5 min (Fig. 3A, inset), a separate set of experiments was performed (Fig. 3B) with sample collections at 2 and 3 min as well. These experiments confirmed that the initial rate of 45Ca\(^{2+}\) uptake was reduced by elevated [Mg\(^{2+}\)]\(_{o}\). The Na\(^{+}\)/Ca\(^{2+}\) exchange inhibitor DCB (10 μM, added at the onset of reoxygenation) also significantly reduced the influx velocity of 45Ca\(^{2+}\) (Fig. 3, A, inset, and B).

Effect of increased [Mg\(^{2+}\)]\(_{o}\) on Ca\(^{2+}\) efflux during reoxygenation. To study the effect of elevated [Mg\(^{2+}\)]\(_{o}\) (5 mM, added at reoxygenation) on Ca\(^{2+}\) efflux, cells were loaded for 90 min with 45Ca\(^{2+}\) (60 min of hypoxia and 30 min of reoxygenation). The buffer was then diluted 100-fold by centrifugation and resuspension. The fractions of cellular 45Ca\(^{2+}\) (of start value) remaining after 95, 120, and 180 min were 84, 74, and 40% and 96, 73, and 41% in the cells exposed to hypoxia-reoxygenation in the absence and presence of increased [Mg\(^{2+}\)]\(_{o}\), respectively, showing similar efflux kinetics (Fig. 4A). Normoxic control cells loaded with 45Ca\(^{2+}\) for 90 min and subjected to buffer dilution also showed the same pattern of decline of isotope content in the presence of increased [Mg\(^{2+}\)]\(_{o}\) (5 mM) added at 60 min as in the presence of normal [Mg\(^{2+}\)]\(_{o}\) (0.8 mM; Fig. 4B).

\([\text{Ca}^{2+}]_{i}\) during hypoxia and reoxygenation and effect of increased [Mg\(^{2+}\)]\(_{o}\). The relationship between \([\text{Ca}^{2+}]_{i}\) and fluorescence intensity of fluo 3 in calcimycin-permeabilized cells is shown in Fig. 5. The relationship was time dependent,
approaching a stable level after 180 min of permeabilization. Figure 6A, inset, shows that fluo 3 fluorescence in calcimycin-permeabilized cells was not influenced by varying [Mg2+]o. Compared with normoxia, fluo 3 fluorescence intensity was increased 4.5-fold at the end of 60 min of hypoxia, corresponding to an increase in [Ca2+]i from 60 to 300 nM (Fig. 6A). The fluorescence signal of fluo 3 rapidly fell at reoxygenation and by the end of experiments was ~30% higher than control cells, corresponding to a [Ca2+]i of 125 nM in reoxygenated cells and 90 nM in normoxic cells, respectively. Increasing [Mg2+]o to 5 mM at the onset of reoxygenation did not affect the fluo 3 signal during reoxygenation (Fig. 6B). The hypoxic group, which received DCB at the onset of reoxygenation, tended to have ~30–40% lower fluo 3 signal than control hypoxiareoxygenation; however, the difference was not statistically significant. Ouabain-treated cells exposed to hypoxia and reoxygenation showed a similar increase in fluo 3 fluorescence signal at the end of 60 min of hypoxia as without ouabain, but no significant fall in fluo 3 fluorescence was achieved during reoxygenation. Elevated [Mg2+]o during reoxygenation of hypoxic and ouabain-treated cells had no effect on fluo 3 fluorescence.

Fig. 4. Effect of increased [Mg2+]o, at the onset of reoxygenation on Ca2+ efflux. Cells were exposed to 1 h of hypoxia and subsequent reoxygenation. Cells were loaded with 45Ca2+ from start of experiments, and the content of 45Ca2+ was determined after diluting extracellular 45Ca2+ at 30 min of reoxygenation. A: cells exposed to 1 h of hypoxia and subsequent reoxygenation with 0.8 (●) and 5 mM [Mg2+]o (○). Values are given as the cellular content of 45Ca2+ in counts per minute (cpm) per milligram of protein. Each point represents the mean of 7–14 determinations from 4 different cell isolations in A and 2–4 determinations from 2 different cell isolations in B.

Effect of increased [Mg2+]o, in hypoxia and reoxygenation and effect of increased [Mg2+]o. The relationship between [Mg2+]o, and fluorescence intensity of MgG in calcimycin-permeabilized cells is shown in Fig. 7A. The relationship approached a stable level after 180 min of permeabilization. Figure 7A, inset, shows that MgG fluorescence in calcimycin-permeabilized cells is less sensitive to increasing Ca2+ than to increasing Mg2+ concentrations. MgG fluorescence intensity was increased twofold compared with normoxic control cells at the end of hypoxia (60 min), showing an increased [Mg2+]o at the end of the hypoxic period (Fig. 7B). The fluorescence signal of MgG started to fall at the onset of reoxygenation and by the end of the experiments was ~20% higher than in normoxic control cells. In cells exposed to hypoxiareoxygenation in the presence of 5 mM [Mg2+]o, mean MgG fluorescence intensity tended to be higher, but no significant difference was found.

Effect of increased [Mg2+]o, in combination with clonazepam or cyclopiazonic acid on Ca2+ accumulation. The effect of clonazepam and cyclopiazonic acid on cell Ca2+ was tested to investigate the possible role of the mitochondria and SR during reoxygenation. Clonazepam (100 μM, added at start) reduced the elevation in cell Ca2+ by 85% at 2 h and 70% at 3 h. Increased [Mg2+]o, significantly increased the inhibitory effect on Ca2+ accumulation when combined with clonazepam (Fig. 8A). The effect of cyclopiazonic acid (SR Ca2+-ATPase inhibitor) on hypoxiareoxygenation-mediated Ca2+ accumulation was also studied. Surprisingly, cyclopiazonic acid (20 μM, added at start) significantly amplified the reoxygenation-induced increase in cell Ca2+ by 45% at 180 min compared with hypoxiareoxygenation without cyclopiazonic acid. This cyclopiazonic acid-induced increase in cell Ca2+ was abolished by increased [Mg2+]o (5 mM, added at reoxygenation). Actually, the cells treated with the cyclopiazonic acid and Mg2+ had significantly lower values than cells exposed to hypoxiareoxygenation without treatment (Fig. 8B).

Effect of increased [Mg2+]o, clonazepam, and their combination on ΔΨm during reoxygenation. The ratio of red to green fluorescence of JC-1 showed linear dose dependence up to 20 μM JC-1 after 10 min of normoxic incubation (R2 = 0.953, linear regression). Cyanide (5 mM) reduced markedly the red-to-green fluorescence ratio of JC-1 after 10 and 60 min of...
normoxic incubation with JC-1, as shown in Fig. 9. The effect of clonazepam (100 μM, added at start) and increased [Mg]$^{2+}$ (supplemented at reoxygenation) on Dc$_m$ was investigated in cells exposed to hypoxia and reoxygenation using the JC-1 fluorescence ratio. Reoxygenated cells had a 24% lower red-to-green fluorescence ratio than normoxic cells, indicating a fall in Dc$_m$. Cells exposed to hypoxia and reoxygenation in the presence of 5 mM [Mg]$^{2+}$ showed a significantly higher (17 ± 3%) red-to-green fluorescence ratio of JC-1 than control hypoxia-reoxygenation-treated cells. Reoxygenated cells in the presence of 100 μM clonazepam (added at start of experiments) showed 40% higher and the combination of clonazepam and elevated [Mg]$^{2+}$ showed a 50% higher red-to-green fluorescence ratio of JC-1, respectively, compared with control reoxygenated cells (Fig. 10A). The red-to-green fluorescence ratio of JC-1 was inverse correlated ($R^2 = 0.975, P < 0.01$) to cell Ca$_{2+}$ in hypoxia-reoxygenation alone or after treatment with Mg$^{2+}$, clonazepam, or the combination of both agents as shown in Fig. 10B.

Cell size and granularity. Forward scatter (FSC) and side scatter (SSC) were determined by flow cytometry to characterize the effect of [Mg]$^{2+}$ and clonazepam on cell size and granularity. Elevated [Mg]$^{2+}$ did not alter FSC (Fig. 11A) or SSC signals (Fig. 11B); however, clonazepam and the combination of clonazepam and elevated Mg$^{2+}$ showed signific-
Fig. 8. A: effect of increased [Mg\textsuperscript{2+}]\textsubscript{o}, clonazepam, and their combination on Ca\textsuperscript{2+} accumulation. Cell Ca\textsuperscript{2+} in normoxic control cells (●) and cells exposed to 1 h of hypoxia and 2 h of reoxygenation with no supplements (●) or with 5 mM [Mg\textsuperscript{2+}]\textsubscript{o}, (●), 100 μM clonazepam (●), and 5 mM [Mg\textsuperscript{2+}]\textsubscript{o}, in combination with 100 μM clonazepam (○) are shown. [Mg\textsuperscript{2+}]\textsubscript{o}, was increased at the onset of reoxygenation, and clonazepam was added from start. Each point represents the mean ± SE of 22–24 determinations from 4 different cell isolations. *P < 0.05 vs. hypoxia-reoxygenation (●) and †P < 0.05 vs. hypoxia-reoxygenation with clonazepam (●) at the respective incubation times by ANOVA and the LSD method. B: effect of increased [Mg\textsuperscript{2+}]\textsubscript{o}, cyclopiazonic acid, and their combination on Ca\textsuperscript{2+} accumulation. Cell Ca\textsuperscript{2+} in control cells (●) and cells exposed to 1 h of hypoxia and 2 h of reoxygenation with no supplements (●) or with 5 mM [Mg\textsuperscript{2+}]\textsubscript{o}, (●), 20 μM cyclopiazonic acid (○), and 5 mM [Mg\textsuperscript{2+}]\textsubscript{o}, in combination with 20 μM cyclopiazonic acid (●) are shown. [Mg\textsuperscript{2+}]\textsubscript{o}, was increased at the onset of reoxygenation, and cyclopiazonic acid was added from start. Each point represents the mean ± SE of 10–12 determinations from 3 different cell isolations. *P < 0.05 vs. hypoxia-reoxygenation (●) at the respective incubation times by ANOVA and the LSD method.

Fig. 9. Effect of cyanide (CN; 5 mM) on the red-to-green fluorescence ratio of JC-1 after 10 and 60 min of incubation (cells were incubated with cyanide throughout the indicated time). Each point represents the mean of 2 determinations from 2 different cell isolations.

DISCUSSION

This study was designed to elucidate the mechanism(s) behind the effect of increased [Mg\textsuperscript{2+}]\textsubscript{o}, on Ca\textsuperscript{2+} accumulation during reoxygenation of hypoxic cardiomyocytes. By increasing [Mg\textsuperscript{2+}]\textsubscript{o}, to 5 mM at reoxygenation, Ca\textsuperscript{2+} accumulation was reduced by 40–50%. Ca\textsuperscript{2+} influx was significantly reduced, whereas Ca\textsuperscript{2+} efflux kinetics were unaltered, and [Ca\textsuperscript{2+}]\textsubscript{i}, and [Mg\textsuperscript{2+}]\textsubscript{i}, were not detectably affected. Increased [Mg\textsuperscript{2+}]\textsubscript{o}, also reduced the reoxygenation-induced fall in ΔΨ\textsubscript{m}, assessed by JC-1 fluorescence and potentiated the effect of cyclopiazonic acid on Ca\textsuperscript{2+} accumulation, ΔΨ\textsubscript{m}, FSC, and SSC.

Cell Ca\textsuperscript{2+} in hypoxia and reoxygenation. The marked increase in cell Ca\textsuperscript{2+} after hypoxia and reoxygenation observed in this study is in agreement with previous reports (8, 25, 33). The present results showing rapidly attained equilibrium of \textsuperscript{45}Ca\textsuperscript{2+} in normoxic cells within 2–5 min of addition to the extracellular buffer also are in agreement with the literature (3, 32). The similarity in equilibration pattern of normoxic cells added either 10 min after start of incubation or 2 h after start of incubation indicates that Ca\textsuperscript{2+} influx and efflux mechanisms remained intact at normoxia during the experiment. Also, uptake of \textsuperscript{45}Ca\textsuperscript{2+} added to cells after exposure to 1 h of hypoxia and 1 h of reoxygenation approached the same value of equilibrium (exchangeable) cell Ca\textsuperscript{2+} (at 3 h) as in cells exposed to the same protocol of hypoxia and reoxygenation and with \textsuperscript{45}Ca\textsuperscript{2+} added at the start of the experiment. This finding indicates that \textsuperscript{45}Ca\textsuperscript{2+} equilibrates with the same exchangeable pool of Ca\textsuperscript{2+} also when added after increasing the size of the pool by hypoxia and reoxygenation and that the availability of Ca\textsuperscript{2+} pool for exchange with \textsuperscript{45}Ca\textsuperscript{2+} was maintained. The longer time needed to equilibrate exchangeable Ca\textsuperscript{2+} with \textsuperscript{45}Ca\textsuperscript{2+} in hypoxia- and reoxygenation-treated cells than in normoxia could be due partly to the increase in size of the pool, partly to slower sarcolemmal influx and efflux rates of Ca\textsuperscript{2+}, and
possibly also to slower exchange of Ca\(^{2+}\) between the cytosol and intracellular stores.

**Effect of increased \([\text{Mg}^{2+}]_{o}\) on cell Ca\(^{2+}\) accumulation, Ca\(^{2+}\) influx, and Ca\(^{2+}\) efflux.** The observed 40–50% reduction in reoxygenation-induced Ca\(^{2+}\) accumulation by increased \([\text{Mg}^{2+}]_{o}\) is in agreement with our previous data (34). The effect of increased \([\text{Mg}^{2+}]_{o}\) on Ca\(^{2+}\) accumulation during reoxygenation was similar irrespective of whether \([\text{Mg}^{2+}]_{o}\) was increased from the start of hypoxia or increased at the onset of reoxygenation, showing that the effect of Mg\(^{2+}\) was exerted at reoxygenation and not during hypoxia. Our results showing significant reduction of influx velocity of Ca\(^{2+}\) and no effect on the pattern of decline of \(45\text{Ca}^{2+}\) in cells exposed to hypoxia and subsequent reoxygenation indicate that the reduction of Ca\(^{2+}\) accumulation by increased \([\text{Mg}^{2+}]_{o}\) during reoxygenation was due to reduced influx of Ca\(^{2+}\). Reduction in Ca\(^{2+}\) accumulation during reoxygenation might conceivably be caused by inhibition of sarcolemmal Ca\(^{2+}\) influx mechanisms such as reverse Na\(^{+}/\text{Ca}^{2+}\) exchange, extensively discussed in our recent report (34), and/or inhibition of Ca\(^{2+}\) accumulation in intracellular pool(s), most probably the SR and mitochondria.

**Methodological aspects in measurements of \([\text{Ca}^{2+}]_{i}\) and \([\text{Mg}^{2+}]_{i}\).** A central aspect in the present work is the selectivity of intracellular fluorescence probes for detecting the free ion concentration of Ca\(^{2+}\) and Mg\(^{2+}\) and the actual contamination of signal by other ions than those intended to detect. Fluo 3 is essentially nonfluorescent unless bound to Ca\(^{2+}\), with the maximum fluorescence signal at 1,400 nM Ca\(^{2+}\). Fluo 3 has a \(K_d\) of 390 nM for Ca\(^{2+}\) according to the manufacturer (Molecular Probes) in 100 mM KCl, 10 mM MOPS (pH 7.2), and 0–10 mM Ca\(^{2+}\)-EGTA at 22°C. This value agrees well with the value found based on our calibration curve in permeabilized cells, which was 305 nM. Fluo 3 exhibits an at least 100-fold Ca\(^{2+}\)-dependent fluorescence enhancement (10). Fluo 3 can also bind Mg\(^{2+}\).
magnitude of the Ca\(^{2+}\)-dependent fluorescence increase of fluo 3 is about eightfold greater than the Mg\(^{2+}\)-dependent fluorescence increase in 100 \(\mu\)M Ca\(^{2+}\) and 10 mM Mg\(^{2+}\), respectively (guideline data provided by Molecular Probes). We therefore conclude that Mg\(^{2+}\) was not likely to significantly influence fluo 3 fluorescence in the present experiments. Similarly, MgG can bind both Mg\(^{2+}\) and Ca\(^{2+}\). However, typical physiological Ca\(^{2+}\) concentrations (10 nM—1 \(\mu\)M) do not usually interfere with MgG fluorescence because the affinity of MgG for Ca\(^{2+}\) is low. Leyssens et al. (22) showed that [Mg\(^{2+}\)] can be assessed using MgG and found that the MgG signal was not affected by changes in [Ca\(^{2+}\)] in the range of 100—400 nM evoked by 10 mM caffeine. Additionally, the increase in the MgG signal by the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenyldiazone (FCCP) was neither significantly affected by removing external Ca\(^{2+}\) nor buffering intracellular Ca\(^{2+}\) with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid. Because our measurements of [Ca\(^{2+}\)] showed values of 60 and 300 nM for normoxic and hypoxic cells, respectively, we conclude that our measurement of MgG fluorescence was likely not contaminated by Ca\(^{2+}\) (K\(_{s}\) of MgG for Ca\(^{2+}\) is 6 \(\mu\)M, data reported by Molecular Probes).

Effect of increased [Mg\(^{2+}\)], on [Ca\(^{2+}\)], during hypoxia and reoxygenation. The increase in fluo 3 fluorescence at the end of hypoxia, demonstrating an increase in [Ca\(^{2+}\)], is in agreement with known changes in [Ca\(^{2+}\)], in cardiac tissue during hypoxia (16) and ischemia (37), probably due to ATP depletion and subsequent inability to maintain Ca\(^{2+}\) gradients across sarcolemma and intracellular stores like the SR. The observed level of fluo 3 fluorescence corresponding to 60 nM in normoxic control cells after 1 h of incubation is in accordance with the value from normoxic cells in data presented by Nishida et al. (27) for end-diastolic [Ca\(^{2+}\)], of 72 ± 6 nM in isolated adult rat cardiomyocytes electrically stimulated at 2 Hz. Increased [Ca\(^{2+}\)], at the end of hypoxia and the subsequent fall in [Ca\(^{2+}\)], starting at reoxygenation is in agreement with reported effects of reoxygenation on [Ca\(^{2+}\)]; recovery during reoxygenation (21, 30). The fluo 3 fluorescence stabilized at a level 60% above the normoxic control, indicating permanent disturbance of cellular Ca\(^{2+}\) homeostasis in reoxygenated cells in the present model, compatible with irreversible cell damage also demonstrated by the rounded shape of the cells and the higher proportion of trypan blue-positive cells. Because increased [Mg\(^{2+}\)] did not alter [Ca\(^{2+}\)], the reduction of Ca\(^{2+}\) accumulation by increased [Mg\(^{2+}\)] indicates reduced intracellular Ca\(^{2+}\) storage by the SR and/or mitochondria.

Effect of increased [Mg\(^{2+}\)], on [Mg\(^{2+}\)], during hypoxia and reoxygenation. To our knowledge, this is the first report on whether increased [Mg\(^{2+}\)] alters [Mg\(^{2+}\)], during reoxygenation of isolated hypoxic cardiomyocytes. Our results showing a large increase in [Mg\(^{2+}\)], at the end of hypoxia were probably a consequence of ATP depletion and reduction of the Mg\(^{2+}\)-ATP complex, as shown previously by others (22). Because cells treated with increased [Mg\(^{2+}\)] did not show higher MgG fluorescence during reoxygenation than cells in the presence of normal [Mg\(^{2+}\)], (0.8 mM) and not lower [Ca\(^{2+}\)], as discussed in Effect of increased [Mg\(^{2+}\)], on [Ca\(^{2+}\)], during hypoxia and reoxygenation, it appears that the primary effect of increased [Mg\(^{2+}\)] was not exerted by elevating [Mg\(^{2+}\)], or lowering [Ca\(^{2+}\)]. We speculate that the effect of increased [Mg\(^{2+}\)] could be exerted on extracellular sites such as the sarcosomal transporters of Ca\(^{2+}\) and/or intracellular organelles like the mitochondria and SR, possibly influenced by the extracellular concentration of divalent cations like Mg\(^{2+}\) and Ca\(^{2+}\) via signal transduction pathway(s).
proportion of dead cells (trypan blue-positive cells) and LDH release were increased significantly within 10 min of reoxygenation in the present model (33). We (34) have previously shown that increased \([\text{Mg}^{2+}]_o\), at the onset of reoxygenation reduced LDH release, and the present study showed that clonazepam does the same. Therefore, increased \([\text{Mg}^{2+}]_o\), and clonazepam attenuate disruption of the cell membrane associated with cell necrosis and possibly late apoptosis. FSC and SSC reflect cell size and internal granularity and surface roughness, respectively (31). The decrease in FSC (cell shrinkage) paralleled either by no change or an increase in SSC (membrane blebbing) represents early changes during apoptosis (4). Reoxygenated cells in our model showing reduced FSC and increased SSC had therefore important characteristics of apoptotic cell death. These changes may be related to hypercontracture of cardiomyocytes as an early manifestation of irreversible cell injury (38). This could be due to activation of actin-myosin cycling by restoring of oxygen supply, ATP synthesis at still highly elevated \([\text{Ca}^{2+}]_i\), and mitochondrial \(\text{Ca}^{2+}\) uptake (35). The samples taken at the onset of reoxygenation and analyzed during the following 2–3 min showed a small reduction in FSC and increase in SSC compared with normoxic control cells; however, we assume that this is an immediate response to reoxygenation and not to the hypoxia. Our previous study (33) supports this view by showing that if cells were fixed with paraformaldehyde (1%) at the onset of reoxygenation, they had the same characteristics of apoptotic cell death. These changes may be associated with cell necrosis and possibly late apoptosis. FSC and SSC (membrane blebbing) represents early changes during reoxygenation or reperfusion of the myocardium. Clonazepam and the combination of clonazepam and \(\text{Mg}^{2+}\) attenuated the reoxygenation-induced reduction in FSC and increase in SSC. The combination of clonazepam and \(\text{Mg}^{2+}\) tended to give larger effects on both FSC and SSC. Kang et al. (15) showed that apoptosis during reoxygenation was through a mitochondrion-dependent pathway. As described in *Interrelation between \(\text{Ca}^{2+}\) accumulation and \(\Delta\psi_m\), the decrease in \(\Delta\psi_m\) that has been associated with apoptosis (20) was attenuated by clonazepam and increased \([\text{Mg}^{2+}]_o\) in the present model. These results on FSC/SSC and \(\Delta\psi_m\) support each other and indicate an ant apoptotic effect of clonazepam and increased \([\text{Mg}^{2+}]_o\) in this model. Internucleosomal fragmentation of genomic DNA that still can be defined as a hallmark of apoptosis (29) and activation of caspases during reoxygenation in the presence of increased \([\text{Mg}^{2+}]_o\), need to be addressed in further studies.

In conclusion, increased \([\text{Mg}^{2+}]_o\), led to reduced \(\text{Ca}^{2+}\) influx and reduced \(\text{Ca}^{2+}\) accumulation during reoxygenation of hypoxic cardiomyocytes but did not alter \(\text{Ca}^{2+}\) efflux kinetics. Increasing \([\text{Mg}^{2+}]_o\), during reoxygenation did not lead to a significant change in either \([\text{Ca}^{2+}]_i\), or \([\text{Mg}^{2+}]_o\). Our results fit the hypothesis that increased \([\text{Mg}^{2+}]_o\), reduces cellular \(\text{Ca}^{2+}\) accumulation by inhibiting \(\text{Ca}^{2+}\) influx and reducing \(\text{Ca}^{2+}\) storage by the mitochondria and/or SR. Because increasing \([\text{Mg}^{2+}]_o\), during reoxygenation had protective effect on \(\Delta\psi_m\) and reduced \(\text{Ca}^{2+}\) accumulation similar to that of clonazepam, we suggest that a major protective mechanism for elevated \([\text{Mg}^{2+}]_o\), is to protect mitochondria against \(\text{Ca}^{2+}\) overload and \(\Delta\psi_m\), loss. The effectiveness of \(\text{Mg}^{2+}\) added at the onset of reoxygenation appears to strengthen its potential in postischemic treatment.

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REFERENCES


19. Kowalowski AJ, Naia-da Silva ES, Castillo RF, and Verslues AE. \(\text{Ca}^{2+}\)-stimulated mitochondrial reactive oxygen species generation and permeability transition are inhibited by dibucaine or \(\text{Mg}^{2+}\). *Arch Biochem Biophys* 359: 77–81, 1998.
44. Xu KY, Vandegaer K, and Becker LC. The sarcoplasmic reticulum Ca2+-ATPase is depressed in stunned myocardium after ischemia-reperfusion, but remains functionally coupled to sarcoplasmic reticulum-bound glycolytic enzymes. Ann NY Acad Sci 853: 376–379, 1998.