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Regulation of Magnesium Release by cAMP during Chemical Hypoxia in the Rat Heart and Isolated Ventricular Myocytes

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Chemically induced hypoxia has been shown to induce a depletion of ATP. Since intracellular free Mg^{2+} ($[Mg^{2+}]_i$) appears to be tightly regulated following cellular energy depletion, we hypothesized that the increase in $[Mg^{2+}]_i$ would result in Mg^{2+} extrusion following hormonal stimulation. To determine the relation between Mg^{2+} efflux and cellular energy state in a hypoxic rat heart and isolated myocytes, $[Mg^{2+}]_i$, ATP and Mg^{2+} content were measured by using mag-fura-2, luciferin-luciferase and atomic absorbance spectrophotometry. Mg^{2+} effluxes were stimulated by norepinephrine (NE) or cAMP analogues, respectively. Mg^{2+} effluxes induced by NE or cAMP were more stimulated in the presence of metabolic inhibitors (MI). Chemical hypoxia with NaCN (2 mM) caused a rapid decrease of cellular ATP within 1 min. Measurement of $[Mg^{2+}]_i$ confirmed that ATP depletion was accompanied by an increase in $[Mg^{2+}]_i$. No change in Mg^{2+} efflux was observed when cells were incubated with MI. In the presence of MI, the cAMP-induced Mg^{2+} effluxes were inhibited by quinidine, imipramine, and removal of extracellular Na^+. In addition, after several min of perfusion with Na^+-free buffer, a large increase in Mg^{2+} efflux occurred when Na^+-free buffer was switched to 120 mM Na^+ containing buffer. A similar Mg^{2+} efflux was observed in myocytes. These effluxes were inhibited by quinidine and imipramine. These results indicate that the activation of Mg^{2+} effluxes by hormonal stimulation is directly dependent on intracellular Mg^{2+} contents and that these Mg^{2+} effluxes appear to occur through the Na^+-dependent Na^+/Mg^{2+} exchange system during chemical hypoxia.

Key Words: Magnesium, cAMP, ATP, Heart, Ventricular myocytes, Chemical hypoxia

INTRODUCTION

Mg^{2+} is necessary for various enzymatic reaction and cell functions (Flatman, 1984, 1991). However, little is known about the mechanisms regulating the cytosolic free concentration and Mg^{2+} transport in mammalian cells. Gunther and Vormann (1985, 1986) proposed that a Na^+/Mg^{2+} exchanger is directly involved in Mg^{2+} regulation in erythrocytes. This transporter was clearly defined in erythrocytes. However, the existence of a Na^+/Mg^{2+} exchanger in cardiac cells (Gunther & Vormann, 1992), hepatocytes (Gunther & Hollriegl, 1993), and ascites cells (Wolf et al, 1994) has been only inferred by some investigators. Recently, Romani and Scarpa (1990a, 1990b) observed that appropriate hormonal stimulation (i. e., receptor, cAMP) results in large changes in the total cellular content of Mg^{2+} and in subcellular Mg^{2+} redistribution in cardiac and liver cells. They have shown in perfused heart and liver, in myocytes, and in permeabilized cells that the increase in cAMP prompts a rapid and massive release of mitochondrial Mg^{2+}. Hence, these reports have suggested that the mitochondria can act as a major store for Mg^{2+} and can mobilize Mg^{2+} upon hormonal stimulation (Romani & Scarpa, 1990b).

Rapid substantial changes in $[Mg^{2+}]_i$ have been reported during ischemia or metabolic inhibition (Murphy et al, 1989a; Harman et al, 1990; Silverman et al, 1994). However, these changes may have important functional consequences, particularly in the heart, where Mg^{2+} affects the cell functions as well as the regulation of numerous intracellular enzymes

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(Flatman, 1991) because Mg^{2+} is predominantly present in the cell as Mg^{2+}-ATP^{2-} (Corkey et al, 1986). The binding affinity of Mg^{2+} for ATP is greater than for other adenine nucleotides or inorganic phosphate (Velesco et al, 1973). Hence, the decrease in ATP produced by chemical hypoxia should produce a corresponding increase of $[Mg^{2+}]_i$ (Harman et al, 1990; Silverman et al, 1994). These studies provide evidence suggesting that a reduction in the absolute number of cytosolic Mg^{2+} binding sites, possibly due to hydrolysis of ATP, is responsible for increased $[Mg^{2+}]_i$. Therefore, we hypothesized that the increase of $[Mg^{2+}]_i$ induced by chemical hypoxia would result in Mg^{2+} extrusion from the cell following hormonal stimuli.

The present study examines the effects of cAMP and extracellular Na^+ removal on Mg^{2+} efflux in hypoxic rat heart and isolated ventricular myocytes. We also examines the changes in ATP and $[Mg^{2+}]_i$ during chemical hypoxia.

METHODS

Perfused Heart preparation

Male Sprague-Dawley rats (220~270 g) were anaesthetized with pentobabital sodium (100 mg/kg). Hearts were removed into ice-cold buffer and cannulated via the aorta. Hearts were perfused at a rate of 10 ml/min through a cannula in the Langendorff mode (open system), with a buffer containing (in mM) NaCl 120, KCl 3, $CaCl_2$ 1.2, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 10, $NaHCO_3$ 12, and HEPES 10. The buffer was equilibrated with 95% O_2: 5% CO_2 at 37°C to give a pH of 7.4. After 10 minutes of perfusion, the buffer was replaced to one containing the same reagents but without added $MgCl_2$. After 20 min, the effluent perfusate was collected and hearts were then stimulated by the indicated concentrations of agents, which were directly dissolved in buffer. The perfusate was continuously collected, pooled in test tubes each containing 30 seconds of perfusate, and Mg^{2+} content was then measured by atomic absorbance spectrophotometry (AA, Varian AA-575, USA).

Isolation of cardiac ventricular myocytes

Cardiac ventricular myocytes were prepared according to the procedure of De Young et al (1989) by collagenase digestion from ventricle of rat hearts. Hearts were perfused for several min with bicarbonate-buffered Joklik medium followed by recirculation with Joklik medium supplemented with collagenase (100 U/ml), BSA (10 mg/ml), glucose (10 mM), $NaHCO_3$ (12 mM), and $CaCl_2$ (50 μM). After 25 min at 37°C (pH 7.2), the ventricles were quartered and placed in 25 ml of Joklik medium. The softened tissue was triturated, and the liberated cells were washed three times and suspended in Joklik medium supplemented with 1.25 mM $CaCl_2$, 12 mM $NaHCO_3$, 10 mM glucose, 1% BSA, and 95% O_2-5% CO_2, pH 7.2. $CaCl_2$ was added in 250 μM increments at 10-min intervals until a final concentration of 1.25 mM was reached. Cell viability was assessed by microscopy.

Mg^{2+} transport on cardiac ventricular myocytes

One hour after isolation, myocytes were washed three times and resuspended, at the final concentration of 3~4 mg protein/ml, in a medium containing (mM) NaCl 120, KCl 3, KH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 12, glucose 10, and HEPES 10, pH 7.2 (at 37°C with 95% O_2-5% CO_2) and resuspended in the same medium but without added $MgCl_2$. For studies of Mg^{2+} efflux, cells were incubated, at concentration of 300 μg protein/ml, in the same medium but without added $MgCl_2$. Duplicate 800 μl samples were taken at 0, 3 and 9 min of incubation, and cells were sedimented using an Eppendorf microfuge (1 min at 15,000 rpm). Where indicated, various agents were added to the incubation medium. Mg^{2+} content of the supernate was measured by AA. Another sample was dissolved in 0.2% triton and analyzed for protein by the Bradford technique (1976).

Measurement of ATP in myocytes

To examine the influence of metabolic inhibitor on intracellular ATP levels, myocytes were incubated, at a concentration of 300 μg protein/ml, in the incubation medium. Aliquots of the experimental samples were withdrawn before and 1, 3, 5, 10, 15 and 20 min after the addition of NaCN and rapidly sedimented in microfuge tubes. Mg^{2+} content of the supernate was measured by AA. For the measurement of ATP, the pellets were extracted with 700 μl ice-cold (4°C) 5% perchloric acid. This extract was neutralized with 1 M $KHCO_3$ (4°C) and centrifuged for 14 min in a chilled centrifuge. ATP was assayed by monitoring luminescence of firefly luciferase-catalyzed oxidation of D-luciferin (ATP Assay Kit, Sigma).

Measurement of $[Mg^{2+}]_i$ in myocytes

To examine the relationship between $[Mg^{2+}]_i$ and ATP levels, collagenase-dispersed ventricular myocytes were loaded with 4 μM mag-fura-2 by exposure to the AM ester for 45 min at room temperature, in a Joklik medium supplemented with 1.2 mM $CaCl_2$, 12 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 10 mM glucose, and 1% BSA. The cells were washed two times with Joklik medium that contained 1% BSA and then were resuspended at a concentration of 4×10^4 cells/ml in a regular buffer containing zero $MgCl_2$, pH 7.2. The mag-fura-2 fluoresence was monitored using a dual-excitation wavelength spectrophotometer (Univ. of Pennsylvania Biochemical Ins. Group) in 1 ml aliquots of cell suspension. The excitation and emission wavelengths were 340/380 and 510 nm, respectively. The cells were continually stirred at 37°C for 5 min before addition of agents. The fluorescence ratios (340/380) were converted to $[Mg^{2+}]_i$ by calibration as previously described.

Chemicals and statistics

Collagenase (CLS-II) was from Worthington Biochemicals Corp. Mag-fura-2, AM was from Molecular Probes, Inc. All other chemicals and reagents were from Sigma Chemical Co. All data presented are mean ± SEM. Differences between experiments were evaluated by Student's unpaired *t*-test. A value of $p < 0.05$ was considered statistically significant.

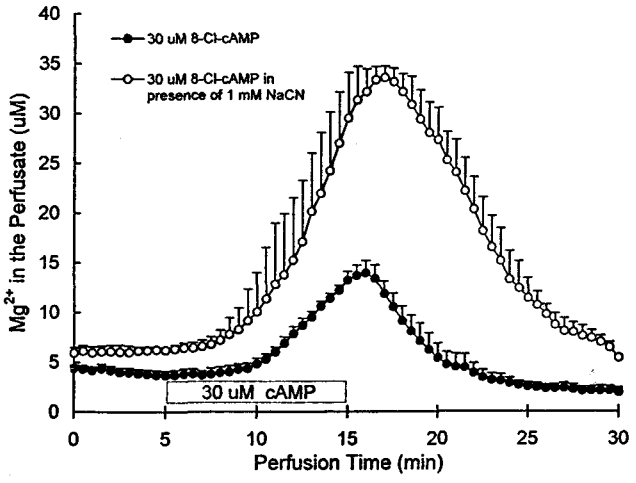

Fig. 1. Potentiation of cAMP-induced Mg^{2+} efflux by NaCN in perfused rat hearts. After 10 minutes of perfusion with a buffer containing 1.2 mM $MgCl_2$, buffer was shifted to one containing zero Mg^{2+}. NaCN was added to the buffer for 5 minutes before samples were collected. Where indicated, 30 μM cAMP was added directly to the perfusate in the absence (●) and in the presence (○) of 1 mM NaCN. Samples were collected every 30 seconds. Data are expressed as means ± SEM (n=4).

RESULTS

Effects of metabolic inhibitors on Mg^{2+} efflux stimulated by cAMP analogues and NE

Romani and Scarpa (1990a, 1990b) showed that Mg^{2+} transport in either direction across the plasma membrane of cardiac and liver cells is under hormonal control. However, these data fall short of characterizing the influence of hormonal stimulation on Mg^{2+} extrusion under the chemical hypoxia, which increases $[Mg^{2+}]_i$. In the present study, the effects of cAMP analogues and NE on Mg^{2+} efflux during chemical hypoxia were evaluated in perfused rat hearts and isolated ventricular myocytes. Fig. 1 shows the results obtained in four hearts in the presence of 1 mM NaCN on perfusate. The addition of 8-(4-chlorophenylthio)-cAMP sodium (8-Cl-cAMP) to the perfusate results in an efflux of Mg^{2+}. Pretreatment of

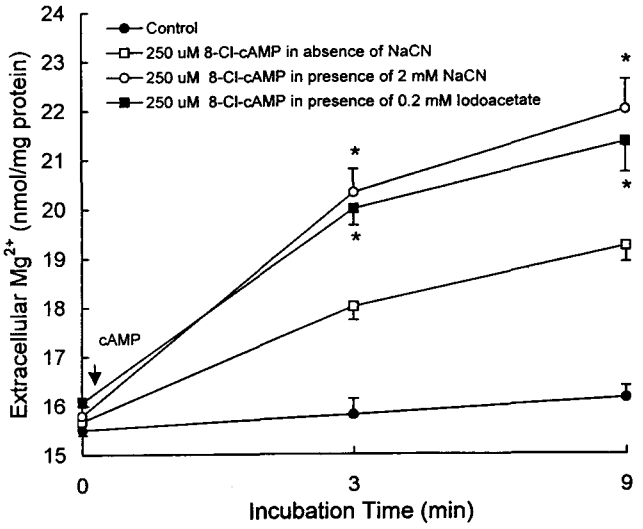

Fig. 2. Potentiation of cAMP-induced Mg^{2+} efflux by metabolic inhibitors in ventricular myocytes. Collagenase-dispersed ventricular myocytes were incubated in a medium containing 1.2 mM $MgCl_2$. Myocytes were incubated, at a concentration of 300 μg protein/ml, in the same medium without $MgCl_2$. After 10 minutes of stabilization, 2 mM NaCN (○, n=6) or 0.2 mM iodoacetate (■, n=5) was added to the medium. After 5 minutes of equilibration, myocytes were stimulated by adding 250 μM 8-Cl-cAMP. Mg^{2+} efflux induced by 250 μM cAMP only (□). Data are means ± SEM of 5 or 9 experments for cAMP only and control. *$p < 0.001$ vs. 8-Cl-cAMP in the absence of NaCN.

Table 1. Effects of NaCN on the 8-Br-cAMP- or noradrenaline-induced Mg^{2+} effluxes in ventricular myocytes

Agents	Extracellular Mg^{2+} Content (nmol/mg protein) Incubation Time (min after addition) 0	3	9
Control	16.98±0.69	17.35±0.82	17.52±0.78
2 mM NaCN	16.69±0.70	17.01±0.72	17.16±0.71
500 μM 8-Br-cAMP	16.38±0.61	18.77±0.76	20.27±0.63
2 mM NaCN+500 μM 8-Br-cAMP	16.57±0.53	21.11±0.36**	23.11±0.33**
10 μM Noradrenaline	17.21±0.75	19.25±0.81	20.28±0.80
2 mM NaCN+10 μM Noradrenaline	16.79±0.71	20.89±0.74*	23.76±0.62**

Experimental conditions were similar to those described in Fig. 2. Data are means±SEM of 6 different experiments. *$p<0.05$, **$p<0.001$ vs. noradrenaline or 8-Cl-cAMP in the absence of NaCN.

hearts with 1 mM NaCN alone did not increase the Mg^{2+} efflux; often a little increase was observed, as shown in Fig. 3. However, pretreatment with NaCN potentiated potentiated the 8-Cl-cAMP-induced Mg^{2+} efflux in perfused hearts. The decrease in ATP should produce an increase in free Mg^{2+} and then this Mg^{2+} may be responsible for Mg^{2+} efflux, depending on the Mg^{2+} gradients and coupling ratio.

As shown in Table 1 and Fig. 2, incubation of the cells in physiological medium did not alter the Mg^{2+} mobilization. Similarly, there was no change in Mg^{2+} efflux during chemical hypoxia. The addition of 8-Cl-cAMP, 8-bromoadenosine-3′, 5′-cyclophosphate sodium (8-Br-cAMP) or NE (which increases cAMP levels) to a myocyte suspension increases Mg^{2+} efflux in a time-dependent manner similar to that observed in perfused hearts. Also these effluxes were more stimulated in the presence of metabolic inhibitors (2 mM NaCN, 0.2 mM iodoacetate) in myocytes (Fig. 2). To assess the effects of metabolic inhibition on changes in both ATP and $[Mg^{2+}]_i$ following chemical hypoxia, the effects of NaCN on cellular ATP concentration and $[Mg^{2+}]_i$ concentration were measured in cardiac myocytes (Fig. 3, 4). After the addition of 2 mM NaCN, ATP decreased from approximately 20 to 13 nmol/ng protein within 1 min (Fig. 3). Since ATP is a major binding site for Mg^{2+}, a consequence of ATP depletion is an increase in free Mg^{2+}. In five experiments, $[Mg^{2+}]_i$ decreased when ventricular myocytes, loaded with mag-fura-2, were exposed to 500 μM 8-Cl-cAMP (Fig. 4A). The addition of 2 mM NaCN to a myocyte suspension increases $[Mg^{2+}]_i$. Under this condition, after several min of exposure to NaCN, the addition of 500 μM 8-Cl-cAMP to the bathing solution resulted in a more marked decrease in $[Mg^{2+}]_i$ (Fig. 4B). Therefore, these results suggested that the cAMP- or NA-induced effluxes of Mg^{2+} were dependent on a rise in the intracellular free Mg^{2+}.

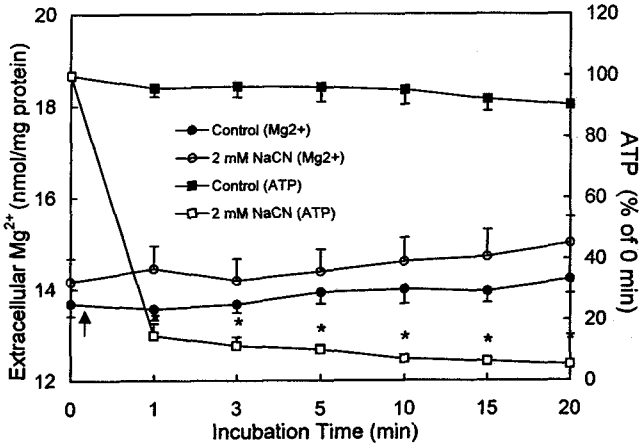

Fig. 3. Effects of NaCN on Mg^{2+} efflux and cellular ATP in ventricular myocytes. Cardiac myocytes were incubated in the medium described in the legend of Fig. 2. Where indicated, 2 mM NaCN was added. Data are means±SEM of 6 different experiments. *$p<0.001$ vs. control (ATP).

Effects of cation modulators and extracellular NaCl on cAMP-induced Mg^{2+} efflux during chemical hypoxia

To investigate whether the specificity of the Mg^{2+} efflux pathway was present in the heart during metabolic inhibition, myocytes and perfused hearts were stimulated with cAMP in the presence of cation modulators and decreasing extracellular Na^+. Fig. 5 shows that when Na^+ was totally replaced isosmotically by choline chloride, cAMP-induced Mg^{2+} efflux was totally abolished during chemical hypoxia. In the presence of 100 μM imipramine, Na^+/Mg^{2+} exchanger inhibitor, the cAMP-stimulated Mg^{2+} efflux was completely depressed, compared with that observed in control medium during metabolic inhi-

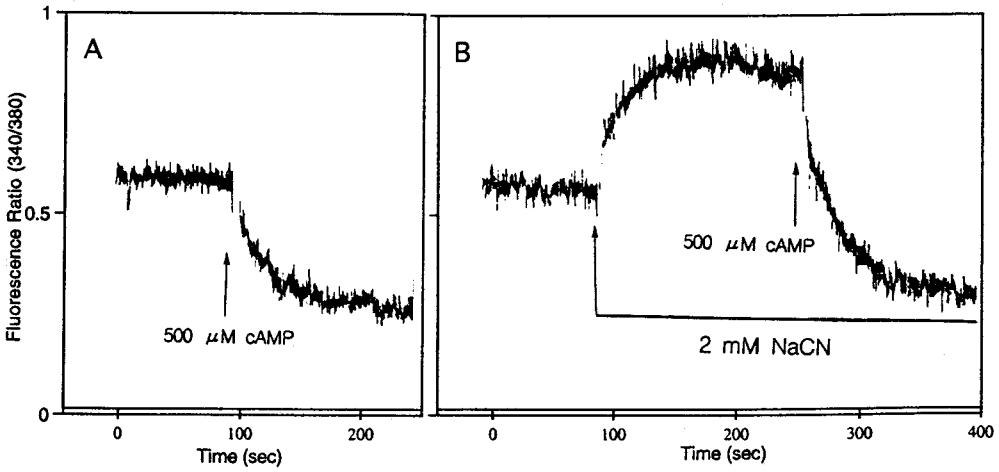

Fig. 4. Potentiation of cAMP-induced a decrease in intracellular Mg^{2+} by NaCN in mag-fura-2-loaded ventricular myocytes. Mag-fura-2-loaded cardiac myocytes were incubated, at a concentration of 4×10^4 cells/ml, in Mg^{2+}- free buffer. After several minutes of stabilization, 500 μM 8-Cl-cAMP was added to the medium at the time indicated by arrow. This figure represents a typical experiment out of five each for the cAMP stimulation in the absence (A) or presence (B) of 2 mM NaCN.

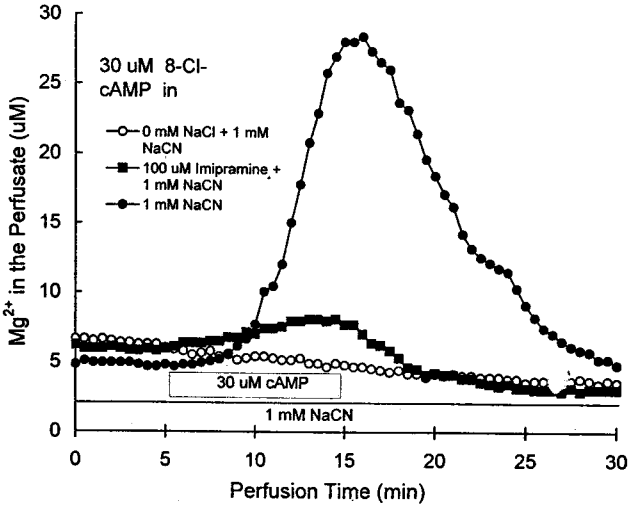

Fig. 5. Influences of removal of extracellular Na^+ or imipramine on the cAMP-induced Mg^{2+} efflux in the presence of NaCN in perfused rat hearts. Where indicated, 30 μM cAMP was added in presence of 1 mM NaCN (●). NaCN (●, ○, ■) and imipramine (■) were added to the buffer for 5 minutes before samples were collected. Na^+-free buffer (○) was perfused for 5 minutes before samples were collected. Experimental conditions were similar to those described in the legend Fig. 1. This figure represents a typical experiment out of three each for the cAMP stimulation in the presence of NaCN and/or zero Na^+, imipramine.

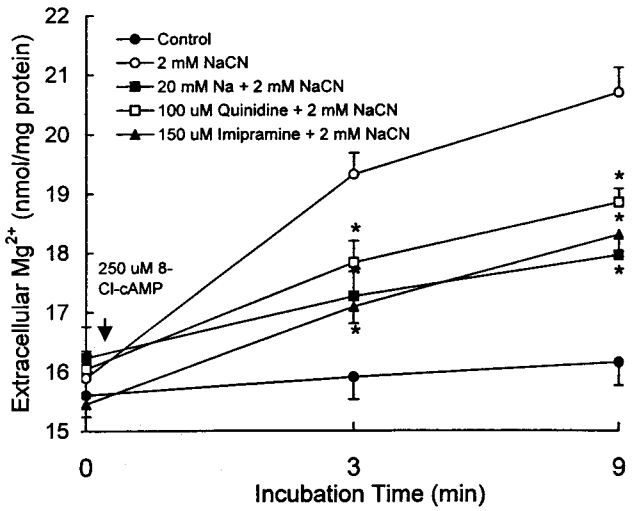

Fig. 6. Influences of quinidine, imipramine, and a reduction in extracellular Na^+ on the cAMP-induced Mg^{2+} efflux in the presence of NaCN in ventricular myocytes. Myocytes were incubated in the medium described in the legend of Fig. 2, in the presence of NaCN and/or quinidine, imipramine, 20 mM Na^+. Where indicated, 250 μM 8-Cl-cAMP was added. Data are means ± SEM of 5 different experiments. *$p < 0.001$ vs. 8-Cl-cAMP in the absence of imipramine or quinidine.

bition. The force of contraction or/and the heart rate were also changed by perfusing hearts with NaCN. Under this condition, no stimulation of Mg^{2+} efflux was observed (data not shown).

To more closely study this effect, the experiments of Fig. 6 were carried out in dispersed ventricular myocytes. Fig. 6 shows that when myocytes were incubated in a medium containing 20 mM Na^+, the cAMP-induced Mg^{2+} efflux was decreased, compared with that observed in the presence of 120 mM Na^+ during chemical hypoxia. The cAMP-induced Mg^{2+} efflux was also decreased in the presence of either

Table 2. Influences of amiloride, benzamil, verapamil, or ryanodine on the 8-Cl-cAMP-induced Mg^{2+} effluxes in the presence of 2 mM NaCN in ventricular myocytes

Agents	Extracellular Mg^{2+} Content (nmol/mg protein) Incubation Time (min after addition) 0	3	9
Control(6)	15.73±0.17	15.95±0.48	16.31±0.57
2 mM NaCN+250 μM 8-Cl-cAMP(8)	15.20±0.66	19.16±0.42	21.35±0.49
200 μM Amiloride+NaCN+cAMP(5)	14.93±0.30	18.99±0.31	20.33±0.24
100 μM benzamil+NaCN+cAMP(5)	15.87±0.44	19.94±0.57	21.81±0.72
500 μM Verapamil+NaCN+cAMP(5)	16.28±0.39	19.57±0.60	21.41±0.62
50 μM Ryanodine+NaCN+cAMP(5)	15.16±0.32	18.95±0.21	20.67±0.25

Number of experiments in parentheses. Experimental conditions were similar to those described in Fig. 6. Data are means ± SEM.

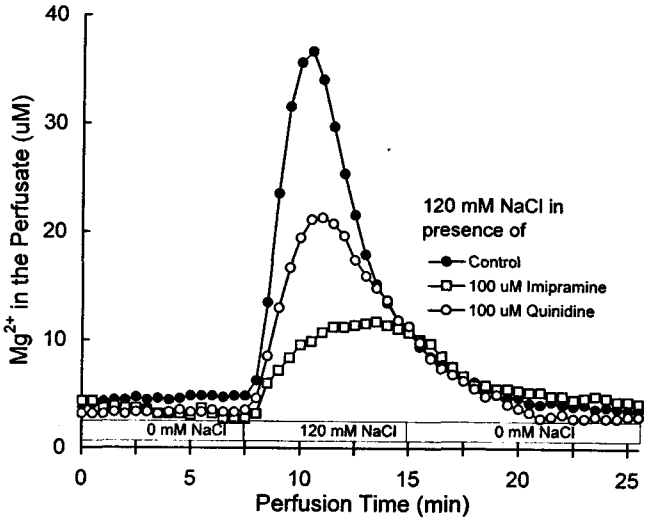

Fig. 7. Effects of an extracellular Na^+ concentration on Mg^{2+} efflux in the absence and presence of imipramine or quinidine in perfused hearts. Hearts were perfused with normal buffer for 7 minutes. After 8 minutes of perfusion with a buffer containing zero Mg^{2+} and 120 mM NaCl, buffer was shifted to another containing zero Mg^{2+} and zero NaCl. After several minutes of perfusion, buffer was shifted to one containing zero Mg^{2+} and 120 mM NaCl (●, control) at the time indicated in the figure. Na^+ was isosmotically substituted by choline chloride. Imipramine (□) or quinidine (○) were directly added to the buffer. Other experimental conditions were similar to those described in Fig. 1. This figure is represents a typical experiment out of three each for control, imipramine and quinidine.

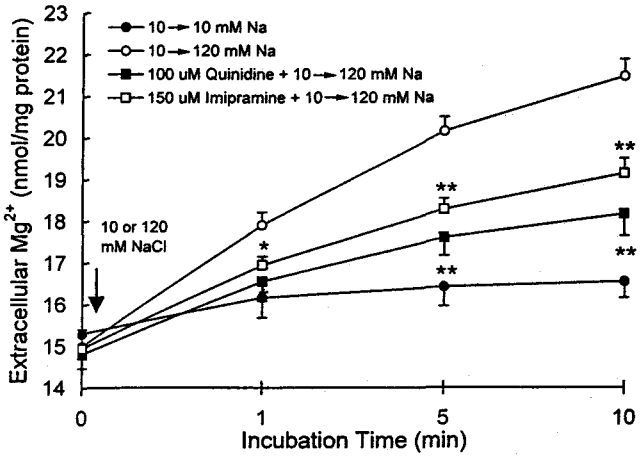

Fig. 8. Effects of an extracellular Na^+ concentration on Mg^{2+} efflux in the absence and presence of imipramine or quinidine in ventricular myocytes. Isolated myocytes were incubated in a buffer containing zero Mg^{2+}, 0.5 mM $CaCl_2$, 10 mM NaCl. After several minutes of stabilization, myocytes were moved into a same buffer (●, 10 mM Na^+) or containing 120 mM Na^+ (○) at the time indicated in the figure. Na^+ was isosmotically substituted by choline chloride. Imipramine (□) or quinidine (■) was directly added in the buffer. Other experimental conditions were similar to those described in Fig. 2. Data are means ± SEM of 5 different experiments. $*p<0.05$, $**p<0.001$ vs. 120 mM Na^+ in the absence of imipramine or quinidine.

quinidine (Na^+ channel blocker) or imipramine (Na^+/Mg^{2+} exchanger inhibitor). By contrast, Table 2 shows that the cAMP-induced increases in Mg^{2+} efflux were not inhibited by 200 μM amiloride (Na^+/H^+ and/or Na^+/Ca^{2+} exchanger inhibitor), 100 μM benzamil (Na^+/Ca^{2+} exchanger inhibitor), 50 μM verapamil (Ca^{2+} channel blocker), or by 50 μM ryanodine (Ca^{2+} transporter inhibitor) during chemical hypoxia. These results suggest that the cAMP-induced Mg^{2+} efflux is not involved in Ca^{2+} transporter, whereas a Na^+-dependent transport mechanism may be involved during chemical hypoxia.

Effects of Na^+ on Mg^{2+} efflux

Another protocols were used to assess the contribution of Na^+ to the observed changes in Mg^{2+} efflux. It has been reported that perfusion of heart cells with Na^+-free solution evokes an increase in $[Mg^{2+}]_i$. Therefore, the effect of extracellular Na^+ content on Mg^{2+} efflux was evaluated in perfused heart and cardiac myocytes. As shown in Fig. 7, the removal of Na^+ alone did not increase the Mg^{2+} efflux. A large increase in Mg^{2+} efflux occurred when the perfusion medium was shifted to one containing 120 mM Na^+ for 8 min; Mg^{2+} efflux returned to control values upon return to Na^+ free mediun. This increase in Mg^{2+} efflux was inhibited by both 100 μM quinidine and imipramine.

Finally, the effects of rapid changes in Na^+ on Mg^{2+} efflux were examined in cardiac myocytes by pretreating them with 10 mM Na^+ medium. No significant increase in Mg^{2+} efflux was seen following a 10 min exposure to 10 mM extracellular Na^+. Similar to that observed in perfused hearts, changing from 10 min Na^+ medium to 120 mM Na^+ medium increased Mg^{2+} efflux in myocytes. Also in cardiac myocytes, this increase in Mg^{2+} efflux was inhibited by both 100 μM quinidine and 150 μM imipramine (Fig. 8).

DISCUSSION

Despite general recognition of the importance of $[Mg^{2+}]_i$ for physiological cell function (Flatman, 1991; Ito & Ehara, 1987), the concentration of $[Mg^{2+}]_i$ and the processes involved in its regulation have been under debate for many years. It has been reported that a large efflux of cellular Mg^{2+} is inducible in perfused rat livers and hearts upon stimulation with β-adrenergic agonists or by other agents which cause an increase in cAMP within cells (Romani & Scarpa, 1990a, 1990b; Romani et al, 1993; Kang et al, 1998). Although significant amounts of Mg^{2+} can be transported through the cell membrane only if Mg^{2+} movement was coupled with the movement of another ion(s) down its electrochemical gradient (Flatman, 1984), the regulation of Mg^{2+} homeostasis in cardiac muscle is not well understood. A major focus of the present study was to explore the relationship between Mg^{2+} mobilization and $[Mg^{2+}]_i$ in cells in which energy metabolism was altered by the use of metabolic inhibitors.

Under normal circumstances, ATP is nearly saturated with Mg^{2+} (Corkey et al, 1986). During ischemia, ATP is hydrolyzed, and Mg^{2+} is liberated, leading to a rise in $[Mg^{2+}]_i$. This concept is supported by studies performed in single rat ventricular cells by Bowers et al (1992) and by a recent study (Silverman et al, 1994) in which single rat ventricular myocytes were loaded with mag-indo 1 and then exposed to rotenone and carbonyl cyanide *p*-trifluoro-methoxyphenylhydrazone (FCCP). In addition, a large increase in cytosolic free Mg^{2+} has been observed under hypoxia (Harman et al, 1990) or other pathological conditions (Corkey et al, 1986; Spurgeon et al, 1990). The present study used a metabolic inhibitors to ATP depletion. The purpose of this treatment was to increase the $[Mg^{2+}]_i$ and to assess the effects of ATP depletion on cAMP-induced Mg^{2+} efflux. The present study showed that cAMP analogues and NE induced a relatively small increase in Mg^{2+} efflux in perfused hearts and ventricular myocytes. In contrast, in the presence of metabolic inhibitors, the same treatment resulted in a large efflux, suggesting that Mg^{2+} efflux is activated by an elevated $[Mg^{2+}]_i$. To assess the effects of metabolic inhibition on changes in both ATP and $[Mg^{2+}]_i$ following chemical hypoxia, the effects of NaCN on cellular ATP concentration and $[Mg^{2+}]_i$ concentration were measured in myocytes. We found that chemical hypoxia accompanied a decrease in cellular ATP and that ATP depletion is accompanied by an incrase in $[Mg^{2+}]_i$. Because the Mg^{2+} gradient may be responsible as a driving force for Mg^{2+} efflux, increases in Mg^{2+} efflux may also occur under hormonal stimuli in cardiac cells. This result is consistent with the mitochondrial Mg^{2+} homeostasis previously described by other investigators where in vitro administration of uncouplers of the oxidative phosphorylation, such as FCCP, to suspensions of intact hepatocytes (Harman et al, 1990) or sublingual mucous acini (Zhang & melvin, 1992) induces a major increase in cytosolic free Mg^{2+} (Harman et al, 1990; Gunther et al, 1992). In contrast, a release of Mg^{2+} into the extracellular medium of the same magnitude as the Mg^{2+} efflux induced NE (Romani et al, 1991), in which no potentiation of the efflux was observed when the uncouplers and NE were added together, suggesting a similar pool of Mg^{2+} as the target for both agents.

Furthermore, we exposed hearts to Na^+-free medium to test the possibility that Na^+-free medium stimulated the increase in $[Mg^{2+}]_i$. A large increase in Mg^{2+} efflux occurred when Na^+ free medium was switched to one containing 120 mM Na^+. This result supports the notion that the Mg^{2+} gradient in hearts largely reflect changes in Mg^{2+} efflux. This is similar to studies by Murphy et al (1989b), in which $[Mg^{2+}]_i$ was observed to increase more than two-fold when

myocytes were superfused with Na^+-free buffer to reverse the normal Na^+ gradient and encourage cellular Ca^{2+} entry via Na^+-Ca^{2+} exchange. Also, in cultured heart cells, perfusion with Na^+-free buffer increased $[Mg^{2+}]_i$ (Murphy et al, 1989b; Freudenrich et al, 1992). Therefore, during Na^+-free prefusion or incubation, the increase in Ca^{2+} influx and subsequent increase in $[Ca^{2+}]_i$ due to competition between Ca^{2+} and Mg^{2+} for intracellular sites contributes to the increase in $[Mg^{2+}]_i$ (Freudenrich et al, 1992).

Cellular Mg^{2+} appears to be distributed between nuclei, mitochondria and endoplasmic or sarcoplasmic recticulum (Gunther, 1986). In the cytosol, Mg^{2+} exists mainly in the form of a complex with ATP (Corkey et al, 1986; Garfinkel & Garfinkel, 1984), so that only a small percentage of the total Mg^{2+} is free in the cytosol. This cytosolic free Mg^{2+} concentration is in rapid equilibrium with bound Mg^{2+} and is in the submillimolar range (Romani & Scarpa, 1992; White & Hartzell, 1989). Polimeni and Page (1974) also estimated the free cytosolic Mg^{2+} content to be lower than 1 mM, out of a total Mg^{2+} content of approximately 17 mM. Therefore, to maintain such a low $[Mg^{2+}]_i$ requires active Mg^{2+} extrusion mechanism(s). Early report indicated that the plasma membrane of numerous cell types (Gunther & Vormann, 1974; Murphy et al, 1989b; Flatman & Smith, 1990; Gunthet & Hollriegl, 1993), including sublingual acinar cells (Zhang & Melvin, 1995), contains Na^+/Mg^{2+} exchangers which utilize the Na^+ gradient generated by Na^+, K^+-ATPase as the energy source for the extrusion of Mg^{2+} (Frenkel et al, 1989). Wolf et al (1994) also showed that cAMP induces phosphorylation of the plasma membrane Na^+/Mg^{2+} exchanger, eventually leads to an increase of Mg^{2+} efflux in ascites cells. However, little is known about a Na^+/Mg^{2+} exchanger in heart. Using selective microelectrodes, Fry (1986) reported an increase in $[Mg^{2+}]_i$ on perfusion with low extracellular Na^+. Although this is consistent with Na^+/Mg^{2+} exchanger, this increase in $[Mg^{2+}]i$ could also be explained by an increase in Ca^{2+} altering the Mg^{2+} buffering. Thus, although in some tissues there is evidence in support of Na^+/Mg^{2+} exchanger, its existence in heart remains to be established. To estimate the specificity of the Mg^{2+} efflux pathway, we attempted to identify the effect of some agents on hormones-induced Mg^{2+} efflux during chemical hypoxia. In erythrocytes, Mg^{2+} efflux is mediated by two systems, a Na^+-independent pathway and a Na^+/Mg^{2+} exchange system. The Na^+- independent Mg^{2+} pathway appears to predominate in Mg^{2+} efflux (Gunther & Vormann, 1989). The Na^+- independent pathway appears to be stimulated by increases in external Mg^{2+} concentration and inhibited by quinidine, by ATP depletion, and by increasing external Na^+ (Feray & Garay, 1987). In contrast, Na^+-free medium and Na^+/Mg^{2+} exchange inhibitor quinidine blocked ~80% of the $[Mg^{2+}]_i$ decrease in sublingual acinar cells (Zhang & Melvin, 1994). Also in this study Na^+-free medium and quinidine blocked the cAMP-induced Mg^{2+} efflux during chemical hypoxia in hearts or myocytes indicating that Mg^{2+} efflux in hearts is mediated by two systems, a Na^+-dependent pathway and a Na^+/Mg^{2+} exchange system. The NA-induced Mg^{2+} efflux strictly depends on the Na^+ concentration in the medium (Romani et al, 1993). Zhang and Melvin (1996) observed that Mg^{2+} release from an intracellular pool is mediated by a Na^+-dependent Mg^{2+} transport mechanism in salivary acinar cells, indicating that the Na^+-dependent pathway is the mechanism involved in Mg^{2+} efflux.

The observation that imipramine, one of the most effective inhibitors of the transporter (Wolf et al, 1994b), completely inhibits also cAMP-induced magnesium efflux indicates that the same transport system is involved and suggests a direct effect of the second messenger on the Na^+/Mg^{2+} exchanger. This finding is consistent with a complete inhibition of the cAMP-induced Mg^{2+} efflux in ascites cells (Wolf et al, 1994a). Furthermore, in this study, the 120 mM NaCl-induced Mg^{2+} efflux was inhibited by both quinidine and imipramine. Amiloride inhibited Na^+-coupled transport of alanine (Renner et al, 1988) and hexose (Cook et al, 1987). In hepatocytes and erythrocyte, Na^+-dependent Mg^{2+} mobilization was also inhibited by amiloride (Gunther & Vormann, 1985; Gunther & Hollrieg, 1993). Since Mg^{2+} efflux was Na^+ dependent, we tested other Na^+ inhibitor. Surprisingly, we found that the cAMP-induced Mg^{2+} efflux was not inhibited by amiloride (the most widely used inhibitor of putative Na^+/Mg^{2+} exchanger). In MDCK cells, Mg^{2+} influx is mediated by a unique entry pathway which manifests similar characteristics to Ca^{2+} channels (Quamme & Dai, 1990). However, Ca^{2+} channel blocker verapamil did not affect Mg^{2+} efflux from myocytes, indicating that the cAMP-induced Mg^{2+} efflux process is not mediated by these Ca^{2+} channels.

In conclusion, $[Mg^{2+}]_i$ in hearts and cardiac myocytes is markedly altered by metabolic inhibitors, and these changes reflect to a large degree changes in intracellular ATP. The activation of Mg^{2+} efflux by hormonal stimuli was directly dependent on intracellular Mg^{2+} contents, and cAMP-induced Mg^{2+} efflux is mediated by an electroneutral Na^+, Na^+/Mg^{2+}

exchange system during metabolic inhibition. Further investigations are needed to elucidate the underlying mechanism regulating Mg^{2+} efflux during chemical hypoxia.

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