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Mg^{2+} protects adult beating cardiomyocytes against ischaemia

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Abstract. Although Mg^{2+} reduces infarct size in whole heart models of ischaemia/reperfusion, the cardioprotective effect of Mg^{2+} at the cellular level is still a controversial issue. Therefore, we tested whether Mg^{2+} protects cardiomyocytes against ischaemia. To accomplish this aim we used an experimental model of ischaemia that utilises single beating adult cardiomyocytes in which oxygen tension is tightly regulated without the use of oxygen scavengers or metabolic inhibitors. Taking all these into consideration, this model is probably closer to *in vivo* conditions than the majority of previously published cellular models of ischaemia. We found that the addition of extracellular Mg^{2+} (8 mM) increased the survival of cells exposed to ischaemia. As sarcolemma and mitochondria are end-effectors of cardioprotective signalling, we examined whether Mg^{2+} regulates sarcolemmal and mitochondrial events. Mg^{2+} (8 mM) did not affect the whole cell K^+ current as revealed by patch clamp electrophysiology. Experiments with laser confocal microscopy and the mitochondrial membrane potential-sensitive dye, JC-1, showed that Mg^{2+} (8 mM) did not affect ischaemia-induced mitochondrial membrane depolarisation. However, a significantly lower JC-1 ratio was required to kill cells under control conditions than cells treated with Mg^{2+} (8 mM). Based on the obtained data, we conclude that Mg^{2+} protects single beating cardiomyocytes against ischaemia by increasing cellular resistance to the consequences of mitochondrial membrane depolarisation in the cytosol.

Introduction

Magnesium (Mg^{2+}) is a divalent cation essential for numerous cellular functions. Its importance is particularly underscored by the involvement in catalytic mechanisms of many enzymes, including every enzyme that catalyses a reaction requiring a nucleotide co-factor (reviewed in ref. 1). An extracellular application of Mg^{2+} has been shown to reduce infarct size in experimental animals (2,3). The whole heart models of myocardial infarction used to study the effect of Mg^{2+} have provided an overall view of events taking place during ischaemia/reperfusion but one drawback of this approach is that it is more difficult to study specific intracellular mechanisms due to the complexity of the whole heart. This problem could be overcome by studying isolated cardiomyocytes exposed to ischaemia (4). However, experiments at the cellular level have failed to demonstrate that Mg^{2+} protects cardiac cells against metabolic stress (5). This was a surprising finding considering that Mg^{2+} is required for many intracellular events involving cellular survival. As an example, it has been shown that Mg^{2+} activates sarcolemmal and putative mitochondrial ATP-sensitive K^+ (K_{ATP}) channels (6,7). In numerous studies, it has been demonstrated that compounds activating K_{ATP} channels decrease infarct size, mimic ischaemic preconditioning and improve functional and energetic recovery of cardiac muscle following ischaemia (reviewed in ref. 8). If Mg^{2+} activates K_{ATP} channels, as suggested by previous reports (6,7), it is logical to expect that Mg^{2+} acts in a cardioprotective manner.

Therefore, we decided to test whether Mg^{2+} protects cardiomyocytes against ischaemia. To achieve this goal, a single-cell model of ischaemia developed in our laboratory was applied (9). This model utilises single beating adult cardiomyocytes in which oxygen tension is tightly regulated without the use of oxygen scavengers or metabolic inhibitors, which, together, makes this model closer to *in vivo* conditions than the majority of previously published cellular models of ischaemia. Therefore, we took advantage of this experimental model to determine whether Mg^{2+} protects isolated cardiomyocytes, a pure myocardial preparation free of hormonal, neuronal and vascular influences, against ischaemia.

Materials and methods

Isolation of cardiomyocytes. All experiments were performed on ventricular cardiomyocytes isolated from male CD-1 mice using an established enzymatic procedure (9,10). In brief, hearts were retrogradely perfused at 37˚C with medium 199, followed by Ca^{2+}-EGTA-buffered low-Ca^{2+} medium (pCa=7), and finally low-Ca^{2+} medium containing pronase E

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(8 mg/100 ml), proteinase K (1.7 mg/100 ml), bovine albumin (0.1 g/100 ml, fraction V) and 200 μM $CaCl_2$. Ventricles were cut into fragments in the low-Ca^{2+} medium enriched with 200 μM $CaCl_2$. Cells were isolated by stirring the tissue at 37˚C in a solution containing pronase E and proteinase K supplemented with collagenase (5 mg/10 ml). The first aliquot was removed, filtered through a nylon sieve, centrifuged for 60 sec at 300-400 rpm, and washed. Remaining tissue fragments were re-exposed to collagenase, and isolation was continued for 2-3 cycles.

Experimental protocol of cellular ischaemia. Ischaemia of isolated cardiomyocytes was performed as described previously (10,11). Cardiomyocytes were placed into Tyrode's solution without magnesium (136.5 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 5.5 mM HEPES-NaOH, pH 7.4), plated out on glass coverslips and paced to beat by field stimulation (parameters of the stimulation: 5-20 mV depending on cellular threshold, 5 msec, 1 Hz). Beating cardiomyocytes were perfused with Tyrode's solution without magnesium at a rate of 3 ml/min and, under these conditions, the partial pressure of O_2 (PO_2) in perfusate was 140 mmHg (12). To induce ischaemia, ischaemic Tyrode's solution without magnesium (a solution identical to Tyrode's solution except that the KCl concentration was increased to 16 mM, 1 mM 2-deoxyglucose replaced glucose and the pH was decreased to 6.6) was bubbled with 100% argon (PO_2= 20 mmHg). When the effect of magnesium was examined, 8 mM $MgSO_4$ was added into the Tyrode's solution and the ischaemic Tyrode's solution. Cells exposed to this experimental protocol were used for laser confocal experiments.

Laser confocal microscopy. Cell morphology, size parameters and mitochondrial membrane potential were monitored in cells exposed to the experimental protocol described in the previous section. To measure the mitochondrial membrane potential, cells were loaded with JC-1 according to the manufacturer's instructions (Invitrogen) and continously monitored with LSM-510. Fluorescence was imaged at a 488-nm excitation wavelength, and emission was captured at 530 and 590 nm for green and red channels respectively; the JC-1 ratio refers always to the green/red ratio (13). The moment of cell death was defined as the point when the cell became rounded (ratio of diameters <3).

Patch clamp electrophysiology. For conventional whole cell electrophysiology, cells were superfused with extracellular solution containing 100 mM NaCl, 10 mM KCl, 0.1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 20 mM glucose, 50 mM taurine, and 10 mM HEPES-NaOH. When the effect of the increased concentration of Mg^{2+} was examined, the concentration of $MgSO_4$ was increased to 8 mM. Pipettes (resistance 3-5 MΩ) were filled with 140 mM KCl, 1 mM $MgCl_2$, 5 mM ATP, and 5 mM HEPES-KOH (pH 7.3). For all cells monitored, the membrane potential was normally held at -40 mV and the currents evoked by a series of 400-msec depolarising and hyperpolarising current steps (-100 to +80 mV in 20-mV steps) and recorded directly to a hard disk using an Axopatch-200B amplifier, Digidata-1321 interface and

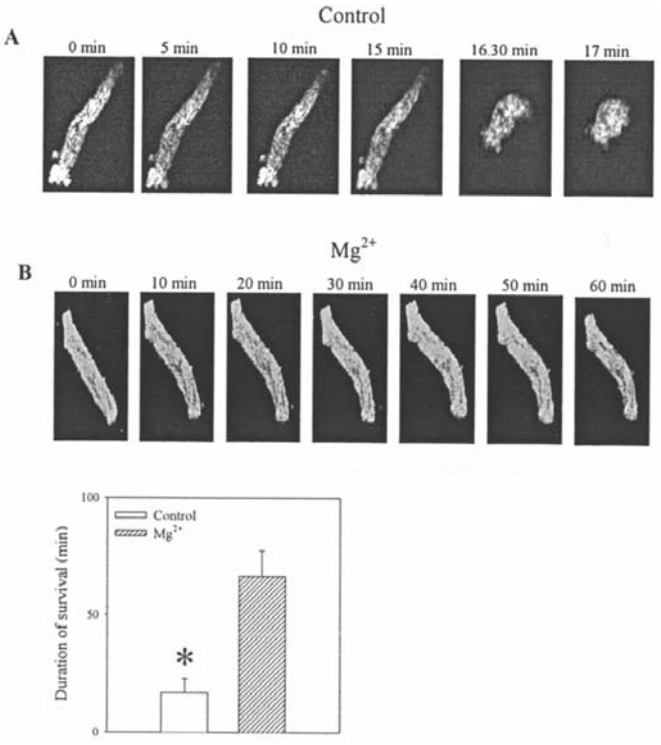

Figure 1. Resistance of cardiomyocytes to ischaemia in the absence (control) and presence of Mg^{2+}. Original images of cardiomyocytes exposed to ischaemia under control conditions (A) and in the presence of Mg^{2+} (8 mM) (B). Lowest panel, average survival time of cardiomyocytes under conditions from A and B. Bars represent the mean ± SEM (n=5-9). *P<0.05.

pClamp8 software (Axon Instruments, Inc., Foster City, CA). The capacitance compensation was adjusted to null the additional whole-cell capacitative current. Currents were low pass filtered at 2 kHz and sampled at 100-μsec intervals (14).

Statistics. Data were presented as the mean ± SEM, with n representing the number of experiments. The difference between the means was assessed using the t-test, or by ANOVA followed by the t-test using the SigmaStat program (Jandel Scientific, Chicago, IL). P<0.05 was considered statistically significant.

Results

The effect of Mg^{2+} on cellular survival in ischaemia. Single beating cardiomyocytes responded to ischaemia by irreversible hypercontracture, which was indicative of cell death (Fig. 1A). The average time of survival in cells exposed to hypoxia was 16.8±5.8 min (n=9). However, when Mg^{2+} (8 mM) was added during ischaemia, cardiomyocytes were able to survive this condition much longer (Fig. 1B). In the presence of Mg^{2+} (8 mM), cells survived 66.5±11.0 min, which was a statistically significant increase in the time of survival when compared to that in the absence of Mg^{2+} (n=5, P<0.001 when compared to the control).

The effect of Mg^{2+} on whole cell current. To determine whether Mg^{2+} affected the activity of K^+ or other channels we

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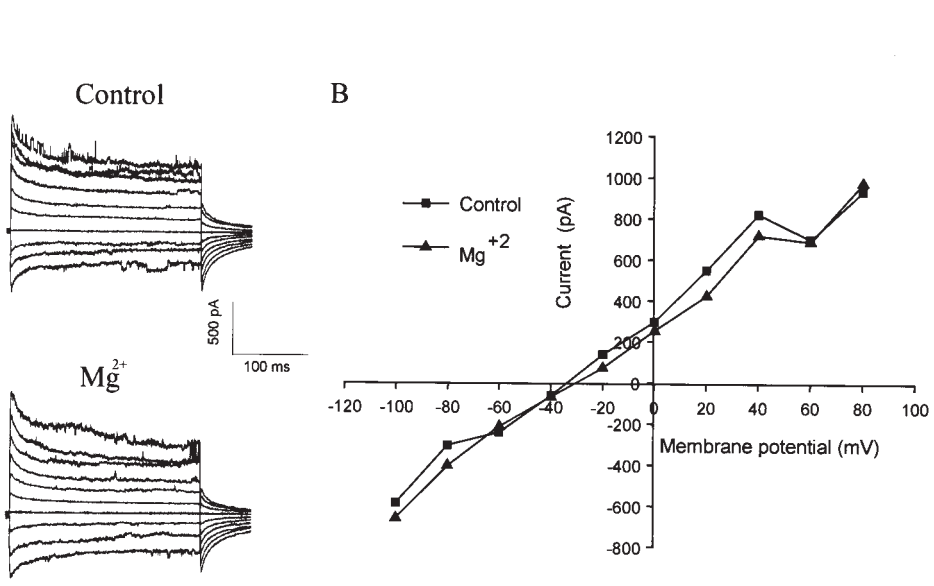

Figure 2. The whole cell K^+ current in cells under control conditions and in the presence of Mg^{2+}. (A) Superimposed membrane currents evoked by identical families of voltage pulses (from -100 to 80 mV; holding potential was -40 mV) in cells under control conditions and after addition of $MgSO_4$ (8 mM). (B) I-V relationship for the experiment in A.

applied whole cell patch clamp electrophysiology on isolated cardiomyocytes. Under control conditions, cells responded to pulses with a series of ion-mediated currents. The whole cell steady-state voltage-current relationship was characterised by inward rectification, which is typical for the absence of active K_{ATP} channels (Fig. 2A and B). Addition of Mg^{2+} did not affect the whole cell current in cardiomyocytes (Fig. 2A and B; at 80 mV the current was 908.4±462.2 and 842.5±345.7 pA under control conditions and after addition of Mg^{2+} respectively; n=3, P>0.05).

The effect of Mg^{2+} on the mitochondrial membrane potential during ischaemia. We and others have previously shown that ischaemia induces mitochondrial membrane depolarisation in beating cardiomyocytes (13,15). It has also been shown that cardioprotection afforded with preconditioning is associated with the induction of mitochondrial membrane depolarisation in the first 20 min of the ischaemic challenge and consequent inhibition of the mitochondrial membrane depolarisation in later stages of ischaemia (15). Stimulation of mitochondrial membrane depolarisation would be also compatible with the activation of putative mitochondrial K_{ATP} channels (16). Therefore, we examined the effect of Mg^{2+} on mitochondrial membrane potential in ischaemia. Under control conditions, ischaemia induced an increase in the JC-1 ratio; in the first 15 min, JC-1 rose from 0.21±0.03 to 0.43±0.11 (n=9, Fig. 3). In the presence of Mg^{2+}, ischaemia had a similar effect on mitochondrial membrane potential as in the absence of Mg^{2+}, i.e. before ischaemia the JC-1 ratio was 0.22±0.08 and 0.60±0.22 after a 15-min-long ischaemia (n=5, Fig. 3). However, cells in the absence of Mg^{2+} died at a significantly lower JC-1 ratio (0.48±0.11, n=9) when compared to the cells in the presence of 8 mM Mg^{2+} (the JC-1 ratio was 0.96±0.21, n=5, P<0.05; Fig. 4).

Discussion

Although Mg^{2+} reduces infarct size in whole heart models of ischaemia/reperfusion (2,17), the cardioprotective effect of Mg^{2+} has not been confirmed at the cellular level (5). In the present study, we showed that Mg^{2+} significantly increased the duration of cell survival during ischaemia demonstrating that this ion does protect the heart against ischaemia by acting on cardiomyocytes.

The mechanism of cardioprotection by Mg^{2+} remains to be elucidated. It has been suggested that an increase in intracellular Mg^{2+} activates sarcolemmal K_{ATP} channels even under normoxic conditions (7). It is well recognised that the activation of these channels protects the heart against ischaemia (18,19). In the present study, Mg^{2+} was cardioprotective when applied extracellularly, and we tested whether such an application of Mg^{2+} would activate sarcolemmal K_{ATP} channels. The results obtained with patch clamp electrophysiology showed that extracellular Mg^{2+} did not regulate the K^+ current suggesting that the opening of sarcolemmal K_{ATP} channels was not involved in Mg^{2+}-mediated cardioprotection.

It has been proposed that the end-effector of cardioprotective signalling could be mitochondria (20). More specifically, it has been suggested that the inhibition of the opening of the mitochondrial permeability transition pore (mPTP) is a crucial event for cellular survival of ischaemia (21). The mPTP is a non-specific megachannel in the inner mitochondrial membrane. The opening of mPTP results in the collapse of the inner membrane potential, uncoupling of respiratory chain, efflux of cytochrome *c* and other molecules which, together, lead to cell death (22). We previously showed that the inhibition of mPTP might be preceded by mild mitochondrial membrane depolarisation (15), which could be ascribed to the activation of putative mitochondrial K_{ATP}

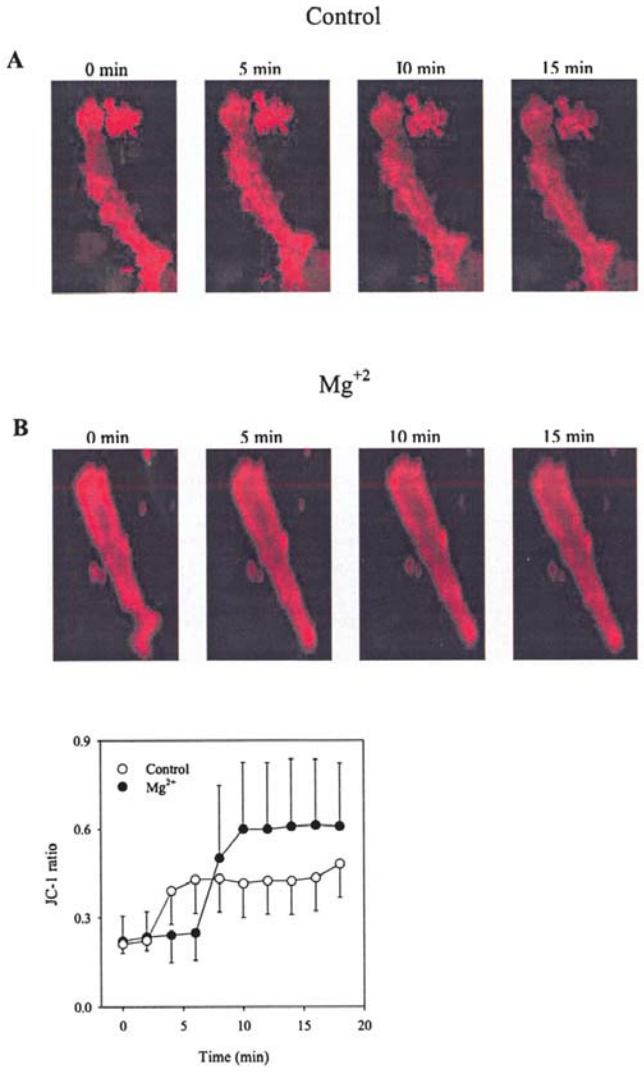

Figure 3. The effect of ischaemia on mitochondrial membrane potential in the absence (control) and presence of Mg^{2+}. Original images of cardiomyocytes loaded with JC-1 in the absence (A) and presence of 8 mM $MgSO_4$ (B). Lowest panel, average time courses corresponding to conditions depicted in A and B. Each point represents the mean ± SEM (n=5-9).

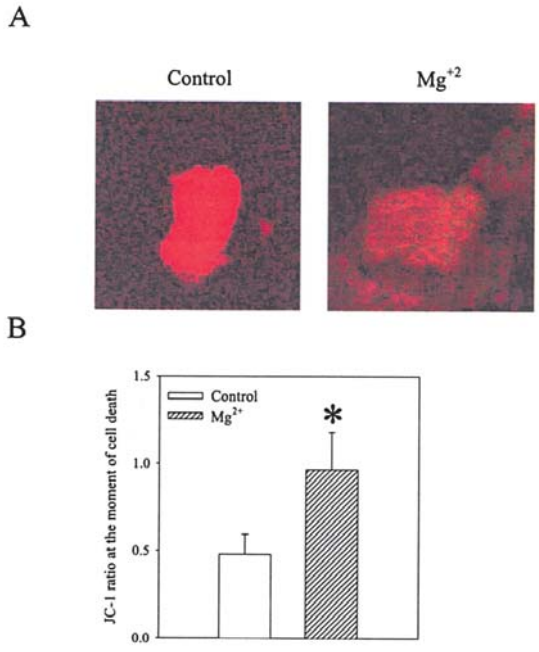

Figure 4. The relationship between mitochondrial membrane depolarisation and cell death in the absence and presence of Mg^{2+}. (A) Original images of cardiomyocytes loaded with JC-1 in the absence (control) and presence of 8 mM $MgSO_4$ at the moment of death. (B) Average values of the JC-1 ratio at the moment of cell death in the absence and presence of Mg^{2+}. Bars represent the mean ± SEM (n=5-9). $^*P<0.05$.

channels (16). On the other hand, Mg^{2+} has been suggested to open putative mitochondrial K_{ATP} channels (23). As the opening of mPTP or any other mitochondrial ion conductance is associated with mitochondrial membrane depolarisation, we measured the mitochondrial membrane potential using JC-1. JC-1 is a cationic dye which exhibits potential-dependent accumulation in mitochondria as indicated by a fluorescence emission shift from green (525 nm) to red (590 nm). Mitochondrial membrane depolarisation is manifest as an increase in the green/red fluorescent ratio (6). In the present study, ischaemia induced gradual mitochondrial membrane depolarisation, which is in accord with previous studies (13,15). The addition of Mg^{2+} did not change the dynamics of the mitochondrial membrane potential during ischaemia suggesting that Mg^{2+} is not cardioprotective due to effects on mitochondrial membrane potential. The main cause of mitochondrial membrane depolarisation in ischaemia is the activation of mPTP, caused by a high intramitochondrial concentration of Ca^{2+}, which induces a series of events leading to the rupture of mitochondrial membranes, the uncoupling of oxidative phosphorylation, releasing numerous proteins and activating degradative enzymes leading ultimately to cell death (22). It is known that Mg^{2+} inhibits voltage-dependent Ca^{2+} channels in cardiomyocytes (reviewed in ref. 3), which could potentially explain the cardioprotection afforded by Mg^{2+}. However, it has been shown that reduction of Ca^{2+} influx by Mg^{2+} is always associated with the prevention of disruption of mitochondrial membrane potential (24). The fact that we did not observe any effect of Mg^{2+} on the mitochondrial membrane potential during ischaemia stands contrary to the idea that the inhibition of Ca^{2+} influx is the main mechanism of Mg^{2+}-induced cardioprotection. The rise in intracellular Mg^{2+} during ischaemia might contribute to the deleterious effect of ischaemia (25). On the other hand, elevated extracellular Mg^{2+} increases Mg^{2+} buffering (26). An increase in Mg^{2+} buffering would result in increased Mg ATP levels which are utilised by ion transporters, such as the Na^+-K^+ pump and the sarcolemmal and sarcoplasmic Ca^{2+} pumps, to maintain intracellular homeostasis (4). The fact that cardiomyocytes were able to sustain prolonged and severe mitochondrial membrane depolarisation in the presence of Mg^{2+} would be compatible with the notion that increased Mg buffering mediates this effect. Research so far has shown that cardioprotective signalling is either associated with end-effectors localised in sarcolemma or mitochondria (reviewed in ref. 27). This study has shown that cardioprotection might be achieved without apparent involvement of the sarcolemma or mitochondria, which seems to be a unique property of Mg^{2+}.

In conclusion, this study has demonstrated that Mg^{2+} protects single beating cardiomyocytes against ischaemia without affecting the whole cell current or the mitochondrial membrane potential. It seems that the protective action of Mg^{2+} is due to the increase in cellular resistance to the consequences of mitochondrial membrane depolarisation.

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