Regulation of L-type calcium current by intracellular magnesium in rat cardiac myocytes

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The effects of changing cytosolic $[Mg^{2+}]$ ($[Mg^{2+}]_i$) on L-type Ca^{2+} currents were investigated in rat cardiac ventricular myocytes voltage-clamped with patch pipettes containing salt solutions with defined $[Mg^{2+}]$ and $[Ca^{2+}]$. To control $[Mg^{2+}]_i$ and cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$), the pipette solution included 30 mM citrate and 10 mM ATP along with 5 mM EGTA (slow Ca^{2+} buffer) or 15 mM EGTA plus 5 mM BAPTA (fast Ca^{2+} buffer). With pipette $[Ca^{2+}]$ ($[Ca^{2+}]_p$) set at 100 nM using a slow Ca^{2+} buffer and pipette $[Mg^{2+}]$ ($[Mg^{2+}]_p$) set at 0.2 mM, peak L-type Ca^{2+} current density (I_{Ca}) was 17.0 ± 2.2 pA pF^{-1}. Under the same conditions, but with $[Mg^{2+}]_p$ set to 1.8 mM, I_{Ca} was 5.6 ± 1.0 pA pF^{-1}, a 64 ± 2.8% decrease in amplitude. This decrease in I_{Ca} was accompanied by an acceleration and a –8 mV shift in the voltage dependence of current inactivation. The $[Mg^{2+}]_p$-dependent decrease in I_{Ca} was not significantly different when myocytes were preincubated with 10 μM forskolin and 300 μM 3-isobutyl-1-methylxanthine and voltage-clamped with pipettes containing 50 μM okadaic acid, to maximize Ca^{2+} channel phosphorylation. However, when myocytes were voltage-clamped with pipettes containing protein phosphatase 2A, to promote channel dephosphorylation, I_{Ca} decreased only 25 ± 3.4% on changing $[Mg^{2+}]_p$ from 0.2 to 1.8 mM. In the presence of 0.2 mM $[Mg^{2+}]_p$, changing channel phosphorylation conditions altered I_{Ca} over a 4-fold range; however, with 1.8 mM $[Mg^{2+}]_p$, these same manoeuvres had a much smaller effect on I_{Ca}. These data suggest that $[Mg^{2+}]_i$ can antagonize the effects of phosphorylation on channel gating kinetics. Setting $[Ca^{2+}]_p$ to 1, 100 or 300 nM also showed that the $[Mg^{2+}]_p$-induced reduction of I_{Ca} was smaller at the lowest $[Ca^{2+}]_p$, irrespective of channel phosphorylation conditions. This interaction between $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ to modulate I_{Ca} was not significantly affected by ryanodine, fast Ca^{2+} buffers or inhibitors of calmodulin, calmodulin-dependent kinase and calcineurin. Thus, physiologically relevant $[Mg^{2+}]_i$ modulates I_{Ca} by counteracting the effects of Ca^{2+} channel phosphorylation and by an unknown $[Ca^{2+}]_i$-dependent mechanism. The magnitude of these effects suggests that changes in $[Mg^{2+}]_i$ could be critical in regulating L-type channel gating.

(Received 4 June 2003; accepted after revision 12 November 2003; first published online 14 November 2003)
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Cytosolic $[Mg^{2+}]$ ($[Mg^{2+}]_i$) in cardiac myocytes appears to be 0.6–1.3 mM (Buri & McGuigan, 1990; Hongo *et al.* 1994; Watanabe & Konishi, 2001) and is largely buffered in the cytosol by a variety of diffusible molecules (e.g. ATP) and proteins (Robertson *et al.* 1981; Fabiato, 1983; Konishi & Berlin, 1993). Changes in $[Mg^{2+}]_i$ can have marked effects on fluxes through ion channels in cardiac myocytes (Agus *et al.* 1989; White & Hartzell, 1989; Agus & Agus, 2001). The first study of $[Mg^{2+}]_i$ effects on L-type Ca^{2+} current (I_{Ca}) in frog myocytes showed that, under appropriate conditions (see below), increasing $[Mg^{2+}]_i$ between 0.3 and 3.0 mM decreased I_{Ca} more than 50% (White & Hartzell, 1988). More recent studies in frog and guinea-pig myocytes have confirmed the marked inhibitory actions of increased $[Mg^{2+}]_i$ on I_{Ca} (Yamaoka & Seyama, 1996*a*,*b*, 1998; Pelzer *et al.* 2001; Yamaoka *et al.* 2002); however, none have shown the large changes of current around physiologically relevant $[Mg^{2+}]_i$ reported by White & Hartzell (1988). These recent results therefore raise the issue of whether $[Mg^{2+}]_i$ is a physiologically important regulator of Ca^{2+} channel function.

Two general mechanisms could explain how Mg^{2+} regulates Ca^{2+} fluxes through L-type channels: alteration of ion permeation and modulation of channel gating

 DOI: 10.1113/jphysiol.2003.048538

properties. Cytosolic Mg^{2+} concentrations up to 10 mM do not decrease divalent cation conductance through single L-type Ca^{2+} channels (Kuo & Hess, 1993; Yamaoka & Seyama, 1998), so that it is unlikely that the reported effects of cytosolic Mg^{2+} on macroscopic I_{Ca} (White & Hartzell, 1988; Agus *et al.* 1989; Yamaoka & Seyama, 1996*a*,*b*, 1998; Pelzer *et al.* 2001) result from block of Ca^{2+} permeation through the channel pore. For this reason, we have focused on mechanisms by which $[Mg^{2+}]_i$ could alter L-type channel gating properties.

L-type Ca^{2+} channel gating is regulated by at least three factors: membrane potential (V_m), cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), and channel phosphorylation (McDonald *et al.* 1994). In this regard, a 10-fold increase in $[Mg^{2+}]_i$ has been shown to produce a small negative shift in the V_m dependence for inactivation of Cd^{2+}-sensitive Ba^{2+} current (Hartzell & White, 1989). Channel phosphorylation state also appears to be important in $[Mg^{2+}]_i$-dependent regulation of I_{Ca}. Earlier studies showed that increased $[Mg^{2+}]_i$ inhibited I_{Ca} most prominently under conditions of high channel phosphorylation (White & Hartzell, 1988; Agus *et al.* 1989). Under basal, presumably low phosphorylation conditions, inhibitory actions of $[Mg^{2+}]_i$ were less marked or not observed. Recent studies (Yamaoka & Seyama, 1998; Pelzer *et al.* 2001) suggest that this less pronounced reduction of I_{Ca} under basal conditions might reflect a shift in inhibitory $[Mg^{2+}]$ from a micromolar to millimolar range when the Ca^{2+} channel is phosphorylated and/or an inability of Mg^{2+} to modulate unphosphorylated channels. Finally, only one study examined how $[Mg^{2+}]_i$ influences Ca^{2+}-dependent regulation of Ca^{2+} channels (Yamaoka & Seyama, 1996*a*), and depending on $[Mg^{2+}]_i$, changes in $[Ca^{2+}]_i$ increased or decreased I_{Ca}. The apparent complexity of these Mg^{2+} actions on I_{Ca} suggests that the mechanisms by which $[Mg^{2+}]_i$ modulates L-type channel gating warrant further study.

The purpose of this study was therefore to determine whether physiologically relevant concentrations of cytosolic Mg^{2+} affect mechanisms, e.g. V_m, Ca^{2+}, and channel phosphorylation, that regulate L-type channel gating. A whole-cell patch-clamp technique was used to measure L-type I_{Ca} density while dialysing cells with a pipette solution containing 40 mM Mg^{2+} buffers (30 mM citric acid and 10 mM ATP) to rapidly control $[Mg^{2+}]_i$ levels. We found that increasing pipette $[Mg^{2+}]$ from 0.2 mM to 1.8 mM suppressed I_{Ca} density by ~70%. This inhibitory effect was enhanced by $[Ca^{2+}]_i$ but inhibited by channel dephosphorylation. However, neither V_m-dependent nor Ca^{2+}/calmodulin-dependent mechanisms appeared to account for the marked reduction of I_{Ca} density by $[Mg^{2+}]_i$.

Portions of this work have appeared previously as a preliminary communication (Wang & Berlin, 2002).

Methods

Cell isolation

Adult rat ventricular myocytes were isolated enzymatically as previously described (Mitra & Morad, 1985) from male Sprague–Dawley rats (200–225 g). Animals received an intraperitoneal injection of sodium pentobarbitone (50–100 mg kg^{-1}), and after full anaesthesia was achieved, a thoracotomy was performed to rapidly remove the heart, in accordance with the procedures approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey. Following isolation, myocytes were stored in a refrigerator and used within 1–8 h.

Measurement of membrane currents

Myocytes were placed in a chamber mounted on an inverted microscope (Nikon Inc., Japan) and superfused with a modified Tyrode solution (see Solutions below). Ca^{2+} current was measured in the whole-cell configuration with patch pipettes having resistances of 1.0–1.5 MΩ when filled with pipette solutions (see below). Outward K^+ current was blocked by Cs^+ and tetraethylammonium ions (TEA) in the pipette solution, while Na^+ current was suppressed by addition of 30 μM tetrodotoxin (TTX) to the Tyrode solution and by depolarizing V_m to –40 mV with ramp pulses prior to test protocols (Nilius *et al.* 1985). All experiments were performed at room temperature. Experiments were conducted when whole-cell voltage clamps had time constants ranging from 100 to 300 μs without series resistance or capacitance compensation. Cell capacitance was estimated by integrating current elicited by 5 mV depolarizations from the holding potential of –70 mV

Experimental protocols

Cells were depolarized every 30 s from a holding potential of –70 mV to –40 mV with a 1 s ramp and then depolarized to a test potential of 0 mV for 200 ms. In some experiments, the *I–V* relationships were also obtained periodically by varying the test potential between –30 and +60 mV (in 10 mV increments) at 0.2 Hz. Displayed current records were obtained after 5 min in the whole-cell

configuration to allow adequate intracellular dialysis (see Results), unless otherwise indicated. Data were analysed using pCLAMP software, version 8.0 (Axon Instruments, Union City, CA, USA), and I_{Ca} was calculated as 200 μM $CdCl_2$-sensitive difference current. Displayed membrane currents are current recordings shown without linear leak subtraction, unless otherwise indicated.

In experiments that monitored indo-1 loading, fluorescence intensity at 410 nm (the isosbestic point of this indicator in our system) was measured during illumination with 360 nm light using methodologies previously described in Konishi & Berlin (1993).

Solutions

The pipette solution was composed of (mM): 100 caesium gluconate, 10 Pipes (caesium salt), 15 TEACl, 0.5 NaH_2PO_4, 0.1 Tris-GTP, 5 EGTA along with ATP, Mg-ATP, citric acid, magnesium citrate, $MgCl_2$ and $CaCl_2$ to produce free $[Mg^{2+}]$ of 0.2, 0.6 and 1.8 mM, pH 7.2, at specified free $[Ca^{2+}]$ of 1, 100 and 300 nM. Free $[Mg^{2+}]$ and $[Ca^{2+}]$ were calculated using a computer program (WinMAXC 2.40 obtained at http://stanford.edu/~cpatton/maxc.html). A second series of experiments was carried out using the same pipette solution with 5 mM BAPTA and 15 mM EGTA. In some experiments, indo-1 (K^+ salt) was added to the pipette solution at a final concentration of 100 μM. The superfusion solution was a modified Tyrode solution containing (mM): 145 NaCl, 4 KCl, 2 $CaCl_2$, 10 Hepes, 1 $MgCl_2$, and 10 glucose, pH 7.4. When noted, $CaCl_2$ in this solution was decreased to 0.5 mM.

Reagents

Unless specified, reagents were obtained from Sigma Chemical Corp. (St Louis, MO, USA). Autoinhibitory peptide (AIP; BioMol, Plymouth Meeting, PA, USA), calcineurin autoinhibitory peptide (CAP; CalBiochem, San Diego, CA, USA), okadaic acid (OA; CalBiochem), protein phosphatase 2A (PP_{2A}; Upstate Biochemicals Inc., Lake Placid, NY, USA), and the potassium salt of indo-1 (Molecular Probes, Eugene, OR, USA) were added directly to pipette solutions. Several reagents purchased from CalBiochem (cyclosporine A, 3-isobutyl-1-methylxanthine (IBMX), KN-93, ryanodine, TTX and W_7) and forskolin (BioMol) were prepared as concentrated stock solutions that were applied to bathing solutions 30 min prior to experiments. When DMSO or MeOH was used as the solvent for stock solutions, the final concentration in experimental solutions was less than or equal to 0.1%, and blank solutions containing 0.1% DMSO or MeOH were also prepared for control experiments.

Data analysis

Data are expressed as means ± S.E.M. for the number of cells indicated. Significance was determined using ANOVA and Student's *t* test in commercial software (SigmaPlot, SPSS Inc., Chicago, IL, USA, and JMP IN, Duxbury, Pacific Grove, CA, USA). A *P* value less than 0.05 was considered statistically significant. The percentage change, confidence intervals (95%) and S.E.M. for current density ratios were calculated using Fieller's Theorem (Goldstein, 1964).

Results

Effect of $[Mg^{2+}]_i$ on calcium current

To assess the effects of $[Mg^{2+}]_i$ on whole-cell I_{Ca}, time diaries of I_{Ca} were recorded for test depolarizations to 0 mV, elicited every 30 s, when cells were voltage-clamped with patch electrodes containing different concentrations of Mg^{2+} ($[Mg^{2+}]_p$) in the presence of 100 nM pipette $[Ca^{2+}]$ ($[Ca^{2+}]_p$). In the myocytes dialysed with 0.2 mM $[Mg^{2+}]_p$, I_{Ca} increased for 1–3 min after patch break-through followed by a long period of rundown before the current finally stabilized approximately 20 min into a 30 min observation period (Fig. 1*A*). The initial increase of I_{Ca} was probably caused by the relief from Mg^{2+} block of I_{Ca} due to the reduction of $[Mg^{2+}]_i$ from a resting level of $[Mg^{2+}]_i$ (~1 mM) to 0.2 mM. The secondary rundown is thought to result from the washout of important cytoplasmic constituents (McDonald *et al.* 1994). In the myocytes dialysed with 1.8 mM $[Mg^{2+}]_p$, I_{Ca} at 30 s after patch break-through was much smaller and, with time, declined faster and to a much lower level than that with 0.2 mM $[Mg^{2+}]_p$. The time diaries show that I_{Ca} with 1.8 mM $[Mg^{2+}]_p$ was 60%, 47%, 37%, 35%, 32%, 31% and 22% of I_{Ca} with 0.2 mM $[Mg^{2+}]_p$ at 30 s, 1 min, 3 min, 5 min, 10 min, 15 min and 30 min after patch break-through, respectively. Representative currents at each of these time points are shown in Fig. 1*B*. Thus, differences in current density changed rapidly during the first 3–5 min after patch break-through and thereafter changed slowly.

The period of rapid change in relative current densities in cells voltage-clamped with 0.2 and 1.8 mM $[Mg^{2+}]_p$ might be an indicator of dialysis from pipette solution to the cytosol. To test this assertion, the time course of indo-1 loading was examined in five cells that, when voltage-clamped, displayed an uncompensated time constant for decay of current during 5 mV depolarizations of 0.3 ms. Indo-1 loading was monitored by measuring

fluorescence intensity at the isosbestic point for this Ca^{2+} indicator. These experiments showed that the time constant for increasing fluorescence intensity was 4 min (Fig. 1*A*, inset). This finding is consistent with our previous work (Berlin & Konishi, 1993). Given these data, in subsequent experiments, we waited for 5 min after patch break-through before I_{Ca} was measured. Within 5 min of establishing a whole-cell patch-clamp, cellular loading of small molecules, such as a fluorescent Ca^{2+} indicator, would be greater than 70% complete. Given the 40 mmol l^{-1} of Mg^{2+} buffers in our patch electrode solutions, this would imply that approximately 25 mmol l^{-1} of exogenous Mg^{2+} buffers, i.e. 20 mM citrate along with 70% of the difference between cytosolic and patch ATP concentrations, would have diffused into the cytosol within this time period. Such a large exogenous buffer concentration should be sufficient to overwhelm endogenous cytosolic Mg^{2+} buffering capacity and thereby gain control of $[Mg^{2+}]_i$.

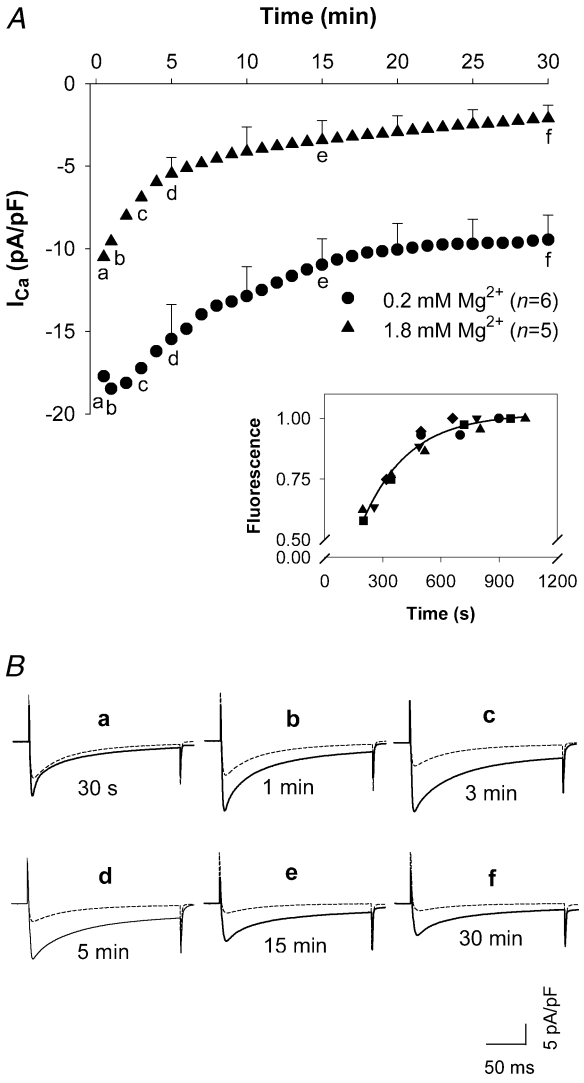

Figure 1. L-type calcium current (I_{Ca}) recorded in rat ventricular myocytes dialysed with low and high $[Mg^{2+}]_p$

A, time diaries of I_{Ca} in rat ventricular myocytes depolarized to 0 mV from a holding potential of –40 mV during dialysis with pipette solutions containing 0.2 mM and 1.8 mM $[Mg^{2+}]_p$. Time 0 coincides with patch break-through. Data are means with S.E.M. displayed for 5, 10, 15, 20, 25 and 30 min time points. The numbers of experiments are indicated in parentheses. Letters (a–f) in each time course correspond to the displayed currents. Inset: time course of indo-1 loading in patch-clamped myocytes. Fluorescence intensity was normalized to the maximal level measured in each of 5 cells (shown with different symbols). The continuous curve is the best exponential function with a time constant of 238 s. *B*, superimposed sample currents, continuous and dashed tracings were recorded with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$, respectively.

The V_m dependence of I_{Ca} was determined by a series of voltage pulses from –30 to +60 mV, as described in Methods. $[Mg^{2+}]_p$ effects on the *I–V* relationship for I_{Ca} are illustrated in Fig. 2*A*. When $[Mg^{2+}]_p$ was increased from 0.2 mM to 0.6 mM (not shown) and 1.8 mM, peak I_{Ca} amplitude was decreased by 56 ± 3.7% (n = 5) and 68 ± 3.5% (n = 5), respectively. Accounting for all experiments, including those where *I–V* relationships were not measured, increasing $[Mg^{2+}]_p$ from 0.2 to 1.8 mM decreased peak I_{Ca} measured at 0 mV by 64 ± 2.8% (n = 10). Increasing $[Mg^{2+}]_p$ also shifted the peak of the *I–V* relationship 5–10 mV in the negative direction and accelerated the rate of current inactivation, as shown by the normalization of current amplitudes in Fig. 2*B*.

Effect of Mg^{2+} on the V_m dependence of calcium current

The observation that a decrease in I_{Ca}, when increasing $[Mg^{2+}]_p$, is accompanied by a shift of the *I–V* relationship towards negative V_m could be interpreted as an effect of $[Mg^{2+}]_p$ on the V_m dependence of Ca^{2+} channel gating. Furthermore, the decrease in I_{Ca} that accompanies this leftward shift in the *I–V* relationship could imply that the V_m dependence of channel inactivation is also shifted to more negative potentials. To examine this possibility, the V_m dependence of channel inactivation was estimated with a two-pulse protocol consisting of a 400 ms prepulse (from –90 to +60 mV in 10 mV increments) followed, after a 3 ms interval at –40 mV, by a 200 ms test depolarization to 0 mV. Figure 3 shows the resulting inactivation curves at 0.2 mM and 1.8 mM $[Mg^{2+}]_p$. With 0.2 mM $[Mg^{2+}]_p$, the curve exhibited a characteristic 'U'-shape, i.e. inactivation reached a maximum with prepulses to 0 mV but decreased with prepulses positive of 0 mV. However, at the higher $[Mg^{2+}]_p$, the degree of current inactivation was more complete at positive V_m. Since V_m-dependent inactivation

of L-type channels requires a long period to reach a pseudo-steady state (Hadley & Lederer, 1991), this protocol was repeated using 3 s prepulses. Results similar to those with 400 ms prepulses were observed. With both protocols, leftward shifts in the inactivation curves were observed. With 400 ms prepulses, increasing $[Mg^{2+}]_p$ from 0.2 to 1.8 mM shifted the V_m for half-maximal inactivation by -8.1 ± 0.7 mV ($n = 5$). The shift was -7.7 ± 1.0 mV ($n = 5$) with 3 s prepulses. These results suggested that $[Mg^{2+}]_p$ can affect the V_m-dependence of channel gating. It should be pointed out that the degree of this shift is small enough that, at –40 mV, the change in Ca^{2+} channel availability is minor, and therefore, a shift in V_m-dependent inactivation is unlikely to account for the marked decrease of I_{Ca} observed with higher $[Mg^{2+}]_p$.

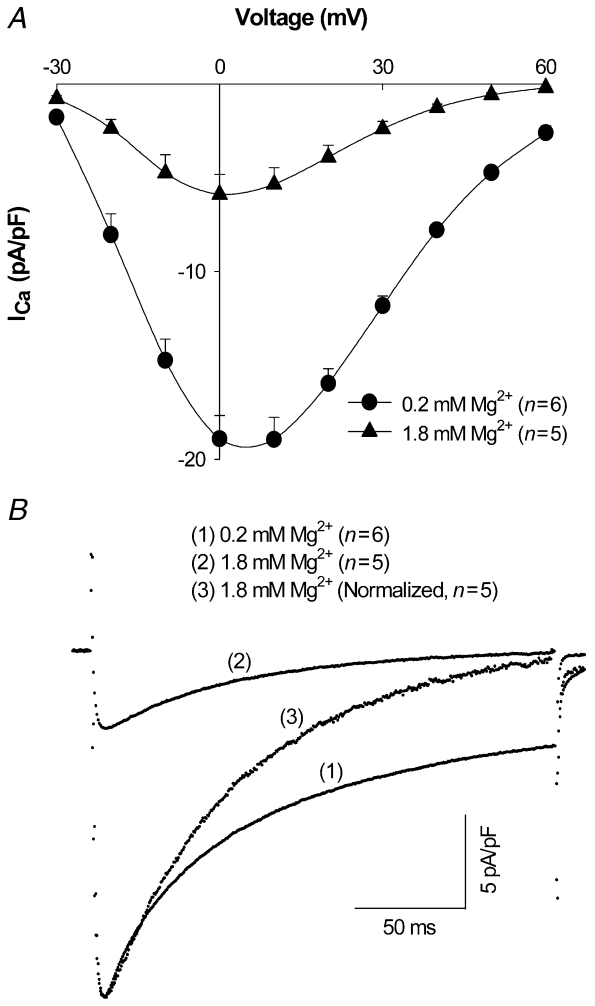

Figure 2. Effect of $[Mg^{2+}]_p$ on I_{Ca}
A, current–voltage relationship of I_{Ca} in myocytes dialysed with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$. Currents were recorded 5 min after establishing the whole-cell patch-clamp configuration. Data are means and S.E.M., with the number of experiments indicated in parentheses. *B*, average I_{Ca} tracings recorded at a test potential of 0 mV in rat ventricular myocytes dialysed with (1) 0.2 mM $[Mg^{2+}]_p$, (2) 1.8 mM $[Mg^{2+}]_p$ and (3) 1.8 mM $[Mg^{2+}]_p$, normalized relative to that with 0.2 mM $[Mg^{2+}]_p$.

Ca^{2+} channel phosphorylation and Mg^{2+} effects on Ca^{2+} current

L-type Ca^{2+} channels are known to be regulated by channel phosphorylation (McDonald *et al.* 1994) and the phosphorylation state of the channel has been reported to be important in determining Mg^{2+} effects on I_{Ca} (White & Hartzell, 1988; Agus *et al.* 1989; Yamaoka & Seyama, 1998; Pelzer *et al.* 2001; Yamaoka *et al.* 2002). To investigate how the modulation of L-type Ca^{2+} channels by $[Mg^{2+}]_p$ is affected by channel phosphorylation, we conducted a series of experiments measuring currents where L-type Ca^{2+} channels were likely to be in phosphorylated and dephosphorylated states, at low (0.2 mM) and high (1.8 mM) $[Mg^{2+}]_p$. To increase channel phosphorylation, cardiac myocytes were first preincubated for 30 min with 10 μM forskolin to activate adenylate cyclase and 300 μM 3-isobutyl-1-methylxanthine (IBMX) to inhibit cAMP and cGMP phosphodiesterases. L-type Ca^{2+} current was then measured 5 min after patch break-through with pipette solutions containing 50 μM okadaic acid (OA) to inhibit protein phosphatases that could dephosphorylate Ca^{2+} channels. This manoeuvre has been found to increase the I_{Ca} in frog and guinea-pig myocytes (Yamaoka & Seyama, 1998; Pelzer *et al.* 2001), presumably by cAMP-mediated phosphorylation of L-type Ca^{2+} channels.

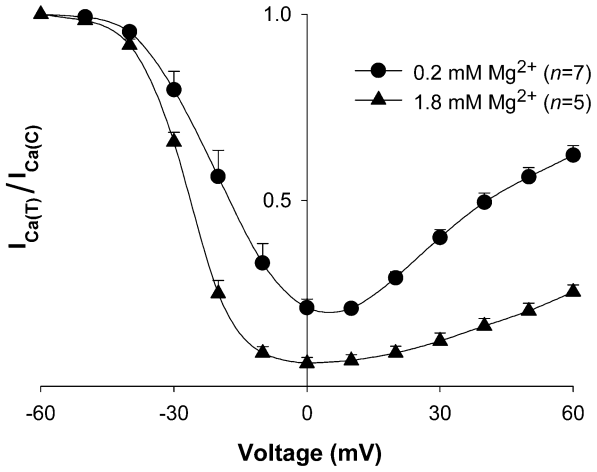

Figure 3. Effect of $[Mg^{2+}]_p$ on inactivation curves for I_{Ca}
The V_m dependence of channel inactivation was measured at 5 min after break-through into the whole-cell patch-clamp configuration with a 2-pulse protocol. I_{Ca} at 0 mV after a given prepulse ($I_{Ca(T)}$) is divided by I_{Ca} at 0 mV after a prepulse to –60 mV ($I_{Ca(C)}$). Data are means and S.E.M., with the number of experiments indicated in parentheses for each $[Mg^{2+}]_p$.

Our experiments showed that in the presence of forskolin, IBMX and OA, I_{Ca} was dramatically increased to densities at 0 mV of 30–40 pA pF^{-1} with 0.2 mM $[Mg^{2+}]_p$ (36.3 ± 2.1 pA pF^{-1}, $n = 3$). To minimize voltage errors due to this high current density, extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was therefore reduced from 2.0 to 0.5 mM. With 0.5 mM $[Ca^{2+}]_o$, I_{Ca} at 0 mV was 16.8 ± 1.4 pA pF^{-1} ($n = 5$) with 0.2 mM $[Mg^{2+}]_p$ in cells exposed to forskolin, IBMX and OA. This current density was not significantly different from that observed under basal conditions with 2.0 mM $[Ca^{2+}]_o$.

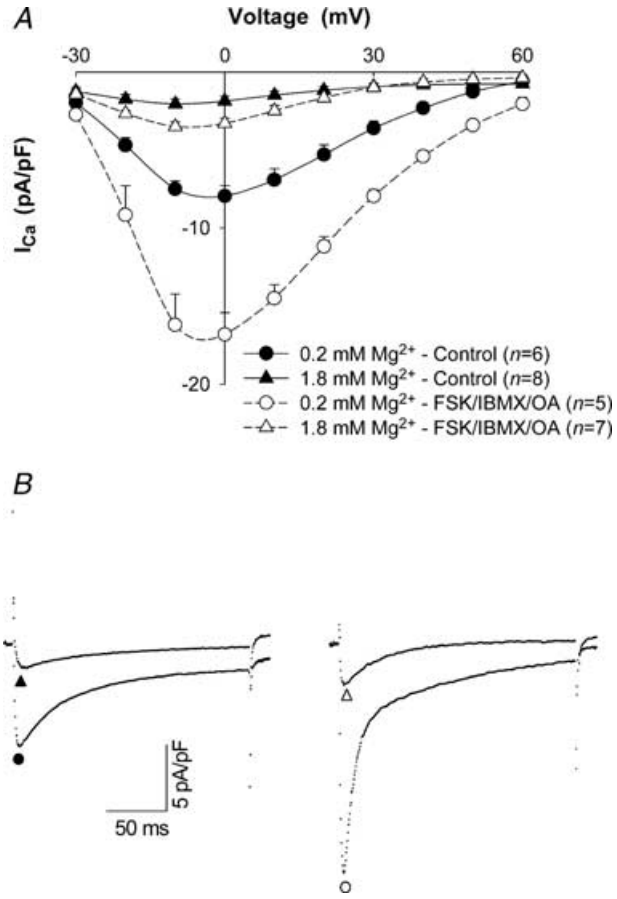

Figure 4. Effect of $[Mg^{2+}]_p$ on I_{Ca} in high phosphorylation conditions

A, current–voltage relationships for I_{Ca} in myocytes dialysed with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$ in control (continuous curves) and high phosphorylation conditions (dashed curves) in the absence and presence of 10 μM forskolin (FSK), 300 μM IBMX and 50 μM OA, respectively, when $[Ca^{2+}]_o$ was set at 0.5 mM. Currents were recorded at 5 min after break-through into the whole-cell patch-clamp configuration. Data are means and S.E.M., with the number of experiments indicated in parentheses. *B*, tracings of typical I_{Ca} records at a test potential of 0 mV in rat ventricular myocytes dialysed with 0.2 mM (●) and 1.8 mM $[Mg^{2+}]_p$ (▲) in control conditions, and 0.2 mM (○) and 1.8 mM $[Mg^{2+}]_p$ (△) in high phosphorylation conditions.

To evaluate the effect of $[Mg^{2+}]_p$ on I_{Ca}, we first repeated experiments in Fig. 2*A* with 0.5 mM $[Ca^{2+}]_o$ (Fig. 4*A* and *B*, left). Under these conditions, increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM decreased peak I_{Ca} amplitude by 75 ± 2.4% ($n = 6$) and shifted the peak of the *I–V* relationship 5–10 mV in the negative direction (Fig. 4*A*, continuous curves). This reduction of I_{Ca} was not statistically different from that observed with 2.0 mM $[Ca^{2+}]_o$, so changing $[Ca^{2+}]_o$ appeared to have no effect on the decrease of I_{Ca} induced by increasing $[Mg^{2+}]_p$. In the presence of forskolin, IBMX and OA, peak I_{Ca} amplitude with 1.8 mM $[Mg^{2+}]_p$ was 3.3 ± 0.4 pA pF^{-1} ($n = 7$). Thus, under these conditions, increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM produced a 79 ± 1.7% inhibition on peak I_{Ca} amplitude (Fig. 4*A*, dashed curves and Fig. 4*B*, right). Additionally, higher $[Mg^{2+}]_p$ caused a –5 mV shift in the peak of the *I–V* relationship. Thus, increasing $[Mg^{2+}]_i$ markedly reduced I_{Ca} under conditions promoting L-type Ca^{2+} channel phosphorylation.

To induce channel dephosphorylation, the catalytic subunit of protein phosphatase 2A (PP_{2A}; 5 units ml^{-1}) was included in the pipette solution. PP_{2A} was chosen because L-type channels are complexed with stoichiometric amounts of PP_{2A} (Davare *et al.* 2000) and PP_{2A} is present in the heart (Herzig & Neumann, 2000). Furthermore, PP_{2A} activity has no requirement for Mg^{2+} and Ca^{2+} (Herzig & Neumann, 2000; Rusnak & Mertz, 2000) so that this enzyme should be insensitive to the experimentally induced changes in cytosolic concentrations of these divalent cations.

The extent of cell dialysis with a protein, such as PP_{2A}, is unknown in our experiments. For this reason, the time course of I_{Ca} was compared in the presence and absence of PP_{2A} (Fig. 5*A* and Fig. 1, respectively). In cells superfused with 2 mM Ca^{2+}-containing Tyrode solution and voltage-clamped with patch pipette solutions containing 0.2 mM $[Mg^{2+}]_p$ and PP_{2A}, the initial rate of current rundown was more rapid than in the absence of PP_{2A}. After 5 min, average I_{Ca} density at 0 mV was 9.8 ± 1.3 pA pF^{-1} ($n = 12$), a level 63 ± 3.7% of that measured in the absence of PP_{2A} (Fig. 5*A*). This decrease in I_{Ca} is statistically significant and is consistent with the results of duBell *et al.* (1996). Representative currents are shown in Fig. 5*A*, inset. Control experiments also showed that adding the enzyme carrier solution to the 0.2 mM Mg^{2+}-containing pipette solution had no effect on I_{Ca} amplitude or kinetics. Thus, a 5-min period of cell dialysis appears to be sufficient for PP_{2A} to produce significant effects on I_{Ca}.

In the presence of PP_{2A}, increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM suppressed peak current by 25 ± 3.4% ($n = 10$). The magnitude of this effect is statistically smaller than that

observed under basal conditions (Fig. 5*B*). Interestingly, with 1.8 mM $[Mg^{2+}]_p$, I_{Ca} density at 0 mV was similar with or without PP_{2A} added to the pipette solution (Fig. 5*B*). In addition, comparing the time diaries in Figs 1 and 5*A* shows that channel dephosphorylation had no significant effect on current magnitude in cells voltage-clamped with patch electrodes containing 1.8 mM $[Mg^{2+}]_p$. These results suggested that channel dephosphorylation reduced Mg^{2+} effects on I_{Ca}.

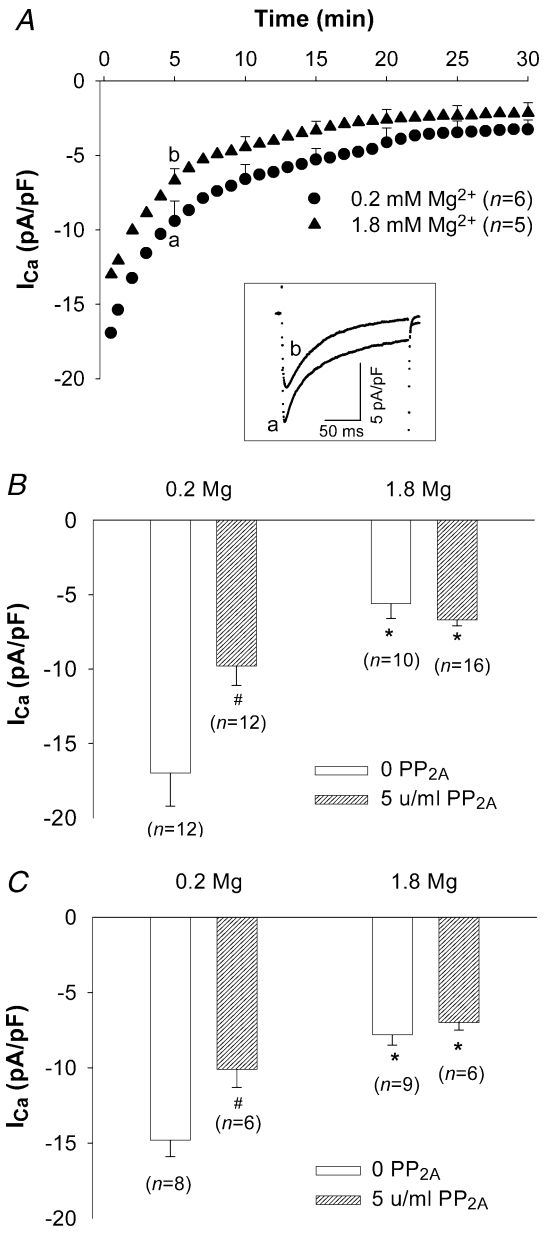

Figure 5. Effect of $[Mg^{2+}]_p$ on I_{Ca} in low phosphorylation conditions

A, time diaries of I_{Ca} in rat ventricular myocytes depolarized to 0 mV from a holding potential of –40 mV during dialysis with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$ electrode solution containing 5 units ml^{-1} PP_{2A}. Data are means with sample S.E.M. displayed for 5, 10, 15, 20, 25 and 30 min time points. The numbers of experiments are indicated in parentheses. Letters (*a* and *b*) in each time course correspond to the sample currents in the inset. Inset: superimposed currents recorded 5 min after patch break-through. *B*, I_{Ca} density in myocytes dialysed with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$ in control (0 PP_{2A}) and low phosphorylation conditions (5 units ml^{-1} PP_{2A}) in the presence of 100 nM $[Ca^{2+}]_p$. *C*, I_{Ca} density in the rat ventricular myocytes dialysed with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$ in control (0 PP_{2A}) and low phosphorylation conditions (5 units ml^{-1} PP_{2A}) in the presence of ~1 nM $[Ca^{2+}]_p$. Currents were measured 5 min after break-through into the whole-cell patch-clamp configuration. Data are means and S.E.M., with the number of experiments indicated in parentheses. Significant changes of I_{Ca}, comparing low (0.2 mM) *versus* high $[Mg^{2+}]_p$ (1.8 mM) and basal (0 PP_{2A}) *versus* low phosphorylation conditions are indicated as * and #, respectively.

$[Ca^{2+}]_i$ and Mg^{2+} effects on calcium current

The experiments above were performed with $[Ca^{2+}]_p$ set to 100 nM, similar to resting levels of $[Ca^{2+}]_i$ in rat myocytes. To evaluate the possible influence of $[Ca^{2+}]_i$ on $[Mg^{2+}]_i$-dependent modulation of current, I_{Ca} was measured at different $[Mg^{2+}]_p$ in nominally Ca^{2+}-free pipette solutions where free $[Ca^{2+}]_p$ was approximately 1 nM. Under these conditions, peak I_{Ca} density at 0 mV in cells voltage-clamped with patch electrodes containing 0.2 mM Mg^{2+} was 14.8 ± 1.1 pA pF^{-1} (n = 8). Increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM altered *I–V* relationships and inactivation of I_{Ca} in a qualitatively similar manner to that with 100 nM $[Ca^{2+}]_p$. However, peak I_{Ca} amplitude was decreased by 45 ± 2.7% (Fig. 6), a significantly smaller reduction of I_{Ca} than observed with 100 nM $[Ca^{2+}]_p$.

When cells were voltage-clamped with patch electrodes containing 300 nM Ca^{2+} and 0.2 mM Mg^{2+}, peak I_{Ca} density at 0 mV was 19.4 ± 1.6 pA pF^{-1} (n = 6). Increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM produced an inhibitory effect on I_{Ca} quantitatively similar to that observed with 100 nM $[Ca^{2+}]_p$ (Fig. 6). These results, summarized in Table 1, suggested that $[Ca^{2+}]_p$ did affect $[Mg^{2+}]_p$ actions on I_{Ca}, particularly when $[Ca^{2+}]_p$ was decreased to very low levels. The following experiments attempted to define how Ca^{2+} and Mg^{2+} might interact in the cell to modulate I_{Ca}.

Effects of intracellular Ca^{2+} fluxes on Mg^{2+} modulation of calcium current

Ca^{2+} influx via L-type channels and sarcoplasmic reticulum (SR) Ca^{2+} release generate local increases in $[Ca^{2+}]_i$ (Stern, 1992; Cheng *et al.* 1993). This local change

in $[Ca^{2+}]_i$ can significantly modulate both the amplitude and macroscopic inactivation kinetics of I_{Ca} in ventricular myocytes (Lacampagne *et al.* 1995; Sham *et al.* 1995; Qu & Campbell, 1998). To assess how $[Ca^{2+}]_i$ participates in the regulation of I_{Ca} by $[Mg^{2+}]_i$, $[Ca^{2+}]_i$ homeostasis was manipulated by two ways: (1) Ca^{2+} release from SR was blocked with 10 μM ryanodine and (2) Ca^{2+} buffering was increased by adding 5 mM BAPTA plus 15 mM EGTA to the pipette solution. In all experiments, $[Ca^{2+}]_p$ was set to 100 nM.

In the presence of 10 μM ryanodine, peak I_{Ca} densities at 0 mV in cells voltage-clamped with $[Mg^{2+}]_p$ of 0.2 mM and 1.8 mM were 16.7 ± 1.6 pA pF^{-1} ($n = 7$) and 4.5 ± 0.9 pA pF^{-1} ($n = 5$), respectively. This change was a 71 ± 3.8% decrease in peak I_{Ca} amplitude (Table 1), a value not significantly different from that observed without ryanodine. Thus, $[Mg^{2+}]_p$ effects on I_{Ca} were not influenced by SR Ca^{2+} release.

To distinguish between local and global effects of Ca^{2+}, slow (EGTA) and fast (BAPTA) Ca^{2+} buffering species were used. Since Ca^{2+} binding kinetics of BAPTA are about 100-fold faster than those of EGTA (Tsien, 1980), Ca^{2+} diffusion distances are quite short (< 100 nm) in the presence of millimolar BAPTA whereas Ca^{2+} diffusion distances can be considerably longer (~1 μm) in the presence of millimolar EGTA (Allbritton *et al.* 1992). With 5 mM BAPTA and 15 mM EGTA included in patch pipette solutions, peak I_{Ca} densities at 0 mV in cells voltage-clamped with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$ were 18.9 ± 1.9 pA pF^{-1} ($n = 6$) and 7.8 ± 0.9 pA pF^{-1} ($n = 6$), respectively. These data show that increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM decreased peak I_{Ca} amplitude by 56 ± 3.5% (Table 1), a change not significantly different than the $[Mg^{2+}]_p$-induced decrease of I_{Ca} with 5 mM EGTA (64 ± 2.8%, $n = 10$). Thus, Ca^{2+} buffers with different kinetics did not affect Mg^{2+} actions on I_{Ca}.

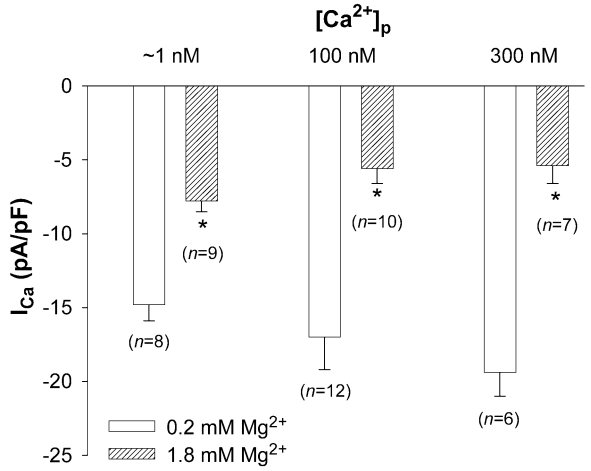

Figure 6. Effect of $[Mg^{2+}]_p$ on I_{Ca} with different $[Ca^{2+}]_p$
Currents were measured at a test potential of 0 mV in myocytes dialysed with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$ at ~1 nM, 100 nM and 300 nM $[Ca^{2+}]_p$. Data are means and S.E.M., with the number of experiments indicated in parentheses. Asterisks (*) indicate significant changes of I_{Ca} between low (0.2 mM) and high $[Mg^{2+}]_p$ (1.8 mM).

Effects of Ca^{2+}/CaM dependent signal-transduction pathways on Mg^{2+} modulation of Ca^{2+} current

The Ca^{2+}-dependence of $[Mg^{2+}]_i$ actions on $I_{Ca,}$ and the inability of Ca^{2+} buffers to alter these actions, point to a possible role for a calmodulin-dependent process in the inhibition of I_{Ca}. To test this hypothesis, the $[Mg^{2+}]_i$ dependence of I_{Ca} density was examined in the presence of various blockers of calmodulin (CaM) and CaM-dependent enzymes.

In cells voltage-clamped in the presence of the CaM inhibitor, W_7 (50 μM), with pipette solutions containing 0.2 mM $[Mg^{2+}]_p$ and 100 nM $[Ca^{2+}]_p$, I_{Ca} was 6.4 ± 0.8 pA pF^{-1} ($n = 5$), a 57 ± 12% decrease compared to I_{Ca} measured in the absence of W_7. The size of this decrease is consistent with a previous report (Caulfield *et al.* 1991). Under the same conditions, except that myocytes were exposed to the CaM-dependent protein kinase (CaMKII) inhibitor, KN-93 (5 μM), in the superfusion solution, I_{Ca} was 9.8 ± 1.2 pA pF^{-1} ($n = 5$), a 37 ± 6% decrease compared to I_{Ca} measured in the absence of this inhibitor. This effect is similar to that reported by Yuan & Bers (1994) with a related CaMKII inhibitor. On the other hand, neither cyclosporin A (CsA, 10 μM in the superfusion solution) nor CAP (10 μM in the pipette solution), inhibitors of CaM-dependent protein phosphatase 2B (PP_{2B}), produced any significant changes of I_{Ca} density in myocytes voltage-clamped with pipette solutions containing 0.2 mM $[Mg^{2+}]_p$ and 100 nM $[Ca^{2+}]_p$.

Comparing I_{Ca} density in the presence of these various blockers with 0.2 mM and 1.8 mM $[Mg^{2+}]_p$ suggested that a CaM-dependent process was not involved in the $[Ca^{2+}]_i$ dependence of $[Mg^{2+}]_i$ actions on I_{Ca}. For example, peak I_{Ca} at 0 mV in cells voltage-clamped with patch electrodes containing 1.8 mM $[Mg^{2+}]_p$ was 3.6 ± 0.7 pA pF^{-1} ($n = 6$) and 2.2 ± 0.4 pA pF^{-1} ($n = 5$) in the presence of KN-93 and W_7, respectively. These current densities represent a 59 ± 5.7% and 60 ± 4.9% decrease in I_{Ca}, respectively, when compared to cells voltage-clamped with electrodes containing 0.2 mM $[Mg^{2+}]_p$ (see Table 1). This degree of current reduction was not significantly different from

Table 1. Effects of experimental manoeuvres on $[Mg^{2+}]_i$-dependent modulation of I_{Ca}

Experimental manoeuvres	Decrease in I_{Ca} density (%)†
Patch electrode Ca^{2+} ($[Ca^{2+}]_p$)	
∼1 nM	45 ± 2.7 (8)*
100 nM	64 ± 2.8 (10)
300 nM	71 ± 3.5 (6)
Change cytosolic Ca^{2+} buffering	
10 μM Ryanodine	71 ± 3.8 (5)
5 mM BAPTA + 15 mM EGTA	56 ± 3.5 (6)
Calmodulin blocker	
50 μM W_7	60 ± 4.9 (5)
Ca^{2+}/calmodulin kinase II blockers	
5 μM KN-93	59 ± 5.7 (6)
100 μM AIP	63 (3)‡
Ca^{2+}/calmodulin phosphatase 2B blockers	
10 μM CsA	56 ± 5.8 (6)
10 μM CAP	56 ± 2.5 (8)

Values of *n* given in parentheses. †Percentage decrease of peak I_{Ca} at 0 mV in cells voltage-clamped with patch electrodes containing 1.8 mM *versus* 0.2 $[Mg^{2+}]_p$, calculated by Fieller's theorem (Goldstein, 1964). ‡The number of replicates is too small to calculate the mean and 95% Confidence Interval of the ratio, and therefore, the S.E.M. using Fieller's theorem. As a result, the ratio of the mean current density with 1.8 mM and 0.2 mM $[Mg^{2+}]_i$ is listed. AIP, autoinhibitory peptide; CAP, calcineurin autoinhibitory peptide. *Significantly different from experiments with $[Ca^{2+}]_p$ equal to 100 nM.

that observed in vehicle-control experiments (i.e. a 64% decrease in I_{Ca}).

Relationship between Ca^{2+} channel phosphorylation and $[Ca^{2+}]_i$ on Mg^{2+}-dependent reduction of I_{Ca}

The experiments above suggested that $[Mg^{2+}]_i$-dependent reduction of I_{Ca} was moderated by reducing channel phosphorylation and $[Ca^{2+}]_i$. The question therefore arises as to whether these two manoeuvres are acting via a common mechanism. To test this possibility, experiments in Fig. 5*B* were repeated except that pipette solutions were prepared without added Ca^{2+} (i.e. ∼1 nM free $[Ca^{2+}]_p$). Under these conditions and with PP_{2A} (5 units ml^{-1}) included in pipette solutions, increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM decreased peak I_{Ca} density from 10.1 ± 1.2 pA pF^{-1} ($n = 6$) to 7.0 ± 0.5 pA pF^{-1} ($n = 6$), a 23 ± 5.5% ($n = 6$) decrease (Fig. 5*C*). In the absence of PP_{2A}, the degree of current reduction on increasing $[Mg^{2+}]_p$ was 45 ± 2.7% ($n = 8$), significantly different from that observed in the presence of PP_{2A}. Even so, by comparing Fig. 5*B* (100 nM $[Ca^{2+}]_p$) and Fig. 5*C* (∼1 nM $[Ca^{2+}]_p$), it was clear that PP_{2A} had similar effects on I_{Ca} amplitude at both low and high $[Mg^{2+}]_p$, irrespective of $[Ca^{2+}]_p$. Thus, these results suggested that increased $[Mg^{2+}]_i$ could block the effects of Ca^{2+} channel phosphorylation on I_{Ca} independently of $[Ca^{2+}]_i$ at or below 100 nM.

Discussion

Experiments in this study demonstrated that increasing $[Mg^{2+}]_p$ around the reported physiological concentration range, 0.6 and 1.3 mM (Buri & McGuigan, 1990; Hongo *et al.* 1994), produced a marked inhibitory modulation of L-type Ca^{2+} current, accelerated current inactivation and caused a negative shift in the V_m dependence of current inactivation. Furthermore, manipulating conditions to favour Ca^{2+} channel dephosphorylation, lessened the degree to which $[Mg^{2+}]_i$ reduced Ca^{2+} current. This modulation was especially pronounced in the presence of $[Ca^{2+}]_p$ (100–300 nM), similar to $[Ca^{2+}]_i$ measured in cells at rest. Even so, the dependence of $[Mg^{2+}]_p$ effects on channel phosphorylation conditions was largely unchanged over the range of $[Ca^{2+}]_P$ tested.

Inhibition of L-type Ca^{2+} current by $[Mg^{2+}]_i$

During the course of whole-cell patch-clamp experiments, I_{Ca} declined faster and to a much lower level in the myocytes dialysed with 0.6 mM or 1.8 mM Mg^{2+}-containing solutions than with solutions containing 0.2 mM Mg^{2+}. At the time when current was routinely measured (5 min after break-through), the amplitude of peak I_{Ca} was 64% smaller in myocytes voltage-clamped with pipette solutions containing 1.8 mM Mg^{2+} than those voltage-clamped with 0.2 mM Mg^{2+}, when $[Ca^{2+}]_p$ was set to 100 nM with 5 mM EGTA in the patch electrode (Table 1). Previous studies have also shown that elevation of $[Mg^{2+}]$ in patch electrode solutions from as low as 1 μM up to 10 mM can dramatically suppress Ca^{2+} current in guinea-pig (Agus *et al.* 1989; Pelzer *et al.* 2001; Yamaoka *et al.* 2002) and frog cardiac myocytes (Yamaoka & Seyama, 1996*a*,*b*, 1998; Yamaoka *et al.* 2002). However, in these previous experiments, changing electrode solution Mg^{2+} concentration around a physiological range of $[Mg^{2+}]_i$ produced considerably smaller changes in I_{Ca} than reported here. These results are, at least in a quantitative sense, different from the present data.

In these previous studies, electrode solutions contained no Mg^{2+} buffers (Agus *et al.* 1989) or weak Mg^{2+} buffering capacity at physiological $[Mg^{2+}]_i$, i.e. 4 mM ATP (Pelzer *et al.* 2001) or 3 mM ATP plus 5 mM EDTA (Yamaoka

& Seyama, 1996*a*,*b*, 1998; Yamaoka *et al.* 2002). This issue is important because cytosolic Mg^{2+} is largely buffered by proteins and small molecules, such as ATP and phosphocreatine (Robertson *et al.* 1981; Fabiato, 1983; Konishi & Berlin, 1993) at concentrations that lead one to question the degree to which $[Mg^{2+}]_i$ was controlled in previous studies, at least around physiological $[Mg^{2+}]_i$. In contrast, the original report investigating $[Mg^{2+}]_i$ regulation of I_{Ca} in frog myocytes (White & Hartzell, 1988) showed that increasing pipette $[Mg^{2+}]$ in the range of 0.3–3.0 mM could significantly decrease I_{Ca}. In their experiments, pipette Mg^{2+} was buffered by 3 mM ATP and 5 mM phosphocreatine, which leads to a relatively higher Mg^{2+} buffering capacity at physiological concentrations. These results, consistent with the present data where 40 mM Mg^{2+} buffers were present in the patch electrode solution, suggest that changes in $[Mg^{2+}]_i$ around a physiological set-point can have large effects in I_{Ca}.

Pipette solution compositions in this study were chosen to provide high Mg^{2+} buffering capacity at physiological $[Mg^{2+}]_i$, while maintaining MgATP in the millimolar range. Mg^{2+} buffering was provided by 30 mM citrate and 10 mM ATP. Since the dissociation constant of citrate for Mg^{2+} is 0.6 mM (calculated from binding constants in Martell & Smith, 1974), this compound should provide strong buffering through the range of $[Mg^{2+}]_p$ used here. A previous report also showed that citrate (10 mM), applied intracellularly, had no effect on I_{Ca} (Hryshko & Bers, 1992). In any case, total citrate concentration in our pipette solutions was constant so that any citrate effects on I_{Ca} should have been systematic in this study.

Membrane potential and $[Mg^{2+}]_i$ effects on I_{Ca}

In addition to markedly reducing I_{Ca} amplitude, increasing $[Mg^{2+}]_p$ shifted the *I*–*V* relationship by 5–10 mV in the negative direction (Fig. 2*A*), a finding consistent with Hartzell & White (1989). Likewise, increasing $[Mg^{2+}]_p$ from 0.2 to 1.8 mM shifted the V_m for half-maximal current inactivation by –8 mV (Fig. 3). This result, coupled with the acceleration of inactivation of I_{Ca} by high $[Mg^{2+}]_p$ (Fig. 2*B*), suggests that increasing $[Mg^{2+}]_p$ promotes Ca^{2+} channel inactivation. The mechanism responsible for the –8 mV shift in current inactivation was not explored in detail; however, two possibilities are obvious. Cytosolic Mg^{2+} could alter the kinetics of a V_m-dependent gating process and/or change surface charge shielding. Regardless of the particular mechanism, the effect of a –8 mV shift in steady state current inactivation would have only a minor effect on channel availability with a holding potential of –40 mV, as indicated in Fig. 3. Therefore, shifts in V_m-dependent channel gating are unlikely to explain the marked reduction of I_{Ca} produced by increasing $[Mg^{2+}]_p$.

Table 2. Effects of $[Mg^{2+}]_i$ on phosphorylation-dependent modulation of I_{Ca} (pA pF^{-1})

	$[Mg^{2+}]_p$ (mM)	
Phosphorylation conditions†	0.2	1.8
Low (PP_{2A})	9.8 ± 1.3 (12)	6.7 ± 0.4 (16)
Basal	17.0 ± 2.2 (12)	5.6 ± 1.0 (10)
High (forskolin, IBMX, OA)	36.3 ± 2.1 (3)	7.6‡

Values of *n* given in parentheses. †$[Ca^{2+}]_i$ set at 100 nM and $[Ca^{2+}]_o$ to 2 mM in all experiments. Currents were measured at 0 mV. ‡This value is extrapolated by reducing the current density (36.3 pA pF^{-1}) measured at 0.2 mM $[Mg^{2+}]_i$ during superfusion with 2 mM $[Ca^{2+}]_o$ by 79%, the reduction in current density measured upon increasing $[Mg^{2+}]_i$ from 0.2 to 1.8 mM with 0.5 mM $[Ca^{2+}]_o$.

Channel phosphorylation and $[Mg^{2+}]_i$ effects on I_{Ca}

Channel phosphorylation state appears to exert a strong influence on Mg^{2+} modulation of I_{Ca} (White & Hartzell, 1988; Agus *et al.* 1989; Yamaoka & Seyama, 1998; Pelzer *et al.* 2001). Furthermore, the effects of increasing $[Mg^{2+}]_i$ on I_{Ca}, i.e. decreased amplitude and accelerated inactivation, are consistent with a decrease in Ca^{2+} channel phosphorylation (Allen & Chapman, 1995; Mitarai *et al.* 2000). Therefore, effects of $[Mg^{2+}]_i$ on I_{Ca} were investigated under conditions strongly favouring or antagonizing L-type channel phosphorylation.

The results of these experiments are quite clear. With 0.2 mM $[Mg^{2+}]_p$, manipulating phosphorylation conditions had a dramatic effect on I_{Ca} density, ranging from a level of 36 pA pF^{-1} in the presence of forskolin, IBMX and OA to less than 10 pA pF^{-1} in the presence of PP_{2A}. Conversely, at 1.8 mM $[Mg^{2+}]_p$, these same manipulations had little effect on current density. These data, compiled or extrapolated from the experiments, are summarized in Table 2.

Viewed in another way, these data suggest that $[Mg^{2+}]_p$ has a much greater modulatory role on I_{Ca} in high phosphorylation conditions (79% reduction) as compared to low phosphorylation conditions (25% reduction). White & Hartzell (1988) also showed that increasing $[Mg^{2+}]_i$ ($[Mg^{2+}]_p$ ranging from 0.3 to 3 mM) in frog ventricular myocytes had a much greater modulatory effect on I_{Ca} in conditions promoting Ca^{2+} channel phosphorylation, consistent with our results. Likewise, preincubation of guinea-pig myocytes with a non-specific kinase blocker, K252, to presumably decrease Ca^{2+} channel

phosphorylation, abolished any effect of $[Mg^{2+}]_i$ on I_{Ca} when cells were voltage-clamped with pipettes containing solutions in which $[Mg^{2+}]$ had been set from 1 μM to 5 mM (Pelzer *et al.* 2001). These results imply that channel phosphorylation is integral to Mg^{2+} actions on I_{Ca}.

Why cytosolic Mg^{2+} should produce a greater effect in conditions that promote channel phosphorylation is unclear. One consistent finding is that cytosolic Mg^{2+} inhibits I_{Ca} with high affinity under basal, presumably low phosphorylation, conditions ($IC_{50} = 4$ μM; Yamaoka & Seyama, 1996*b*); however, under conditions promoting Ca^{2+} channel phosphorylation, the apparent affinity for Mg^{2+} inhibition of I_{Ca} shifts to well over 1 mM (Yamaoka & Seyama, 1998; Pelzer *et al.* 2001; Yamaoka *et al.* 2002). This finding might explain why we observe less pronounced effects of $[Mg^{2+}]_i$ on I_{Ca} in the presence of PP_{2A}, i.e. a $[Mg^{2+}]_i$ of 0.2 mM would produce nearly maximal inhibition of I_{Ca} under dephosphorylating conditions so further increasing $[Mg^{2+}]_i$ would have little additional effect on current. Alternatively, micromolar concentrations of GTP are reported to block $[Mg^{2+}]_i$-dependent effects on I_{Ca} (Yamaoka & Seyama, 1996*b*; Yamaoka *et al.* 2002), but channel phosphorylation is reported to overcome these effects of GTP (Yamaoka & Seyama, 1998). Since our pipette solutions contain 0.1 mM GTP, this second possibility seems quite plausible. In any case, our data establish that physiological $[Mg^{2+}]_i$ is capable of regulating I_{Ca} in the presence of GTP to a degree which is dependent on the level of Ca^{2+} channel phosphorylation.

Two types of molecular mechanisms might explain how Mg^{2+} alters gating kinetics of phosphorylated L-type channels. First, the level of channel phosphorylation might be altered because the activity of several regulatory enzymes, such as adenylyl cyclases (Pieroni *et al.* 1995; Sunahara *et al.* 1996), phosphodiesterases (Sette & Conti, 1996; Percival *et al.* 1997) and phosphatases (Cohen *et al.* 1989; Herzig & Neumann, 2000), are affected by Mg^{2+} at concentrations up to the millimolar range. Second, L-type channel gating has been proposed to be modulated directly by both Mg^{2+} and GTP binding (Yamaoka & Seyama, 1998). Whether one or both of these mechanisms explain Mg^{2+} actions on I_{Ca} can only be determined by direct measurements of Mg^{2+} effects on Ca^{2+} channel phosphorylation.

$[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ effects on I_{Ca}

In the presence of 100 nM $[Ca^{2+}]_p$, increasing $[Mg^{2+}]_i$ from 0.2 to 1.8 mM $[Mg^{2+}]_p$ suppressed the amplitude of I_{Ca} by 64%. However, when $[Ca^{2+}]_p$ was nominally zero (~1 nM), increasing $[Mg^{2+}]_p$ from 0.2 to 1.8 mM decreased the amplitude of I_{Ca} by only 45%, a statistically smaller effect. Conversely, when $[Ca^{2+}]_p$ was increased to 300 nM, the $[Mg^{2+}]_i$-induced reduction of I_{Ca} was not different (71%) from that observed with 100 nM $[Ca^{2+}]_p$ (Table 1 and Fig. 6). These results suggest that the $[Mg^{2+}]_p$ effect is greater in the presence of 100 nM and 300 nM $[Ca^{2+}]_p$ than that with ~1 nM $[Ca^{2+}]_p$ and that, to some degree, the effects of $[Mg^{2+}]_p$ on I_{Ca} are achieved in a Ca^{2+}-dependent manner, i.e. $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ interact to regulate I_{Ca}.

$[Mg^{2+}]_i$ effects on $[Ca^{2+}]_i$ regulation of I_{Ca}

The question then is how $[Mg^{2+}]_i$ affects the $[Ca^{2+}]_i$ regulation of I_{Ca}. Our data (Fig. 6) show that increasing $[Ca^{2+}]_p$ (1–300 nM) tended to increase I_{Ca} at low $[Mg^{2+}]_p$ (0.2 mM) whereas increasing $[Ca^{2+}]_p$ tended to decrease I_{Ca} at high $[Mg^{2+}]_p$ (1.8 mM). These trends in the data suggest that $[Mg^{2+}]_i$ might determine the pattern of $[Ca^{2+}]_i$-dependent regulation of I_{Ca}, e.g. positive or negative regulation of I_{Ca}.

This latter point was not pursued because, given cell-to-cell variability, a demonstration of statistically significant changes in I_{Ca} as a function of $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ would have required many more experiments. Nonetheless, these results are interesting because the reported effects of increasing $[Ca^{2+}]_i$ on I_{Ca} have varied widely in previous studies. Yamaoka & Seyama (1996*a*) have reported that increasing $[Ca^{2+}]_i$ from 10 nM to 1 μM facilitates I_{Ca} in frog myocytes voltage-clamped with electrodes containing either 0.1 or 1 mM Mg^{2+}. At the higher electrode $[Mg^{2+}]$, their results appear opposite of those reported here. No other papers have directly investigated if an interaction between $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ might regulate I_{Ca}. However, many papers have reported that increasing $[Ca^{2+}]_i$ can either facilitate (Bates & Gurney, 1993; Gurney *et al.* 1989; Hirano & Hiraoka, 1994) or inhibit I_{Ca} and single Ca^{2+} channels (Morad *et al.* 1988; Hadley & Lederer, 1991; Hirano & Hiraoka, 1994; You *et al.* 1994). Reviewing these papers does not provide a clear picture about the role of $[Mg^{2+}]_i$ in these changes of I_{Ca}; however, the present results do suggest that changing $[Mg^{2+}]_i$ can affect the manner in which $[Ca^{2+}]_i$, around resting levels, might modulate I_{Ca}.

Possible mechanisms underlying $[Ca^{2+}]_i$ modulation of $[Mg^{2+}]_i$ effects on I_{Ca}

To investigate how $[Ca^{2+}]_i$ is involved in the modulation of I_{Ca} by $[Mg^{2+}]_i$, we manipulated $[Ca^{2+}]_i$ in two ways: blocking SR Ca^{2+} release with ryanodine, and buffering

$[Ca^{2+}]_i$ with the fast Ca^{2+} chelator, BAPTA. With 100 nM $[Ca^{2+}]_p$, increasing $[Mg^{2+}]_p$ from 0.2 to 1.8 mM decreased I_{Ca} amplitude to a similar degree in the presence and absence of 10 μM ryanodine, an indication that SR Ca^{2+} release was not involved in $[Mg^{2+}]_i$ effects on I_{Ca}. Furthermore, the $[Mg^{2+}]_i$-induced decrease of I_{Ca} was similar whether fast (5 mM BAPTA + 15 mM EGTA, 100 nM $[Ca^{2+}]_p$) or slow Ca^{2+} buffer systems (5 mM EGTA, 100 nM $[Ca^{2+}]_p$) were included in the pipette solution. These results indicate that buffering increases of $[Ca^{2+}]_i$, irrespective of the kinetics and capacity of the Ca^{2+} chelator, does not affect Mg^{2+} actions on I_{Ca}, consistent with our observation that increasing $[Ca^{2+}]_i$ with 300 nM $[Ca^{2+}]_p$ also does not significantly change $[Mg^{2+}]_i$-dependent modulation of I_{Ca}. Instead, our data show that $[Ca^{2+}]_i$ must be decreased below 100 nM for the interaction between Mg^{2+} and Ca^{2+} to be observed.

Many potential sites exist on or near the L-type Ca^{2+} channel where Ca^{2+} binding could regulate I_{Ca} (Hering *et al.* 2000; Herzig & Neumann, 2000). Each of these sites is also likely to be a potential site for Mg^{2+} binding. Even so, our experiments focused on a possible role of calmodulin (CaM) for several reasons. First, CaM can bind both Mg^{2+} and Ca^{2+} (Haiech *et al.* 1981) and Mg^{2+} binding to CaM interferes with Ca^{2+}-dependent regulation of enzyme function (Ohki *et al.* 1997). Second, the Ca^{2+} affinity of CaM when it is bound to IQ motif peptides is approximately 50 nM (Black *et al.* 2002), in the same range of Ca^{2+} concentrations used in our pipette solutions. The IQ motif of the L-type channel is located at the C-terminal tail and CaM interaction near this site is thought to participate in channel inactivation and facilitation (Zühlke *et al.* 1999; DeMarla *et al.* 2001). Thus, a reasonable expectation is that Mg^{2+} might modulate this Ca^{2+}-dependent mechanism of channel gating or *vice versa*. To test this hypothesis, the effects of CaMKII inhibitors (KN-93 and AIP), calcineurin inhibitors (CsA and CAP) and the CaM inhibitor W_7 were tested on $[Mg^{2+}]_i$-dependent modulation of I_{Ca}. None of these agents significantly altered the effects of $[Mg^{2+}]_i$ on I_{Ca}. Therefore, our data indicate that a CaM-dependent mechanism does not explain the interaction of $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$ to modulate I_{Ca}. Nevertheless, considering that W_7, probably like other calmodulin blockers, may not produce a specific blockade of calmodulin (Klockner & Isenberg, 1987) and Ca^{2+}/CaM-dependent inactivation (Imredy & Yue, 1994; Victor *et al.* 1997), we cannot entirely rule out the involvement of a Ca^{2+}/CaM-dependent facilitation/inactivation mechanism in the regulation of I_{Ca} by $[Mg^{2+}]_i$.

Participation of $[Ca^{2+}]_i$ in the phosphorylation-dependent regulation of I_{Ca} by $[Mg^{2+}]_i$

Since our results show that $[Mg^{2+}]_i$ effects on I_{Ca} amplitude are dependent on channel phosphorylation, we looked at whether this phosphorylation-dependent regulation of Mg^{2+} effects on I_{Ca} is related in some manner to $[Ca^{2+}]_i$ dependence of Mg^{2+} actions. The effect of increasing $[Mg^{2+}]_p$ was examined under low and basal phosphorylation conditions with ~1 nM and 100 nM $[Ca^{2+}]_p$. With both $[Ca^{2+}]_p$, Mg^{2+} effects were comparable, i.e. under basal phosphorylation conditions, increasing $[Mg^{2+}]_p$ produced a greater decrease in I_{Ca} than in the dephosphorylated channel. Most clearly, high $[Mg^{2+}]_p$ minimized the effect of channel phosphorylation on I_{Ca} with both $[Ca^{2+}]_p$. We interpret these data as suggesting that Mg^{2+} can affect two mechanisms, one phosphorylation-dependent and the other Ca^{2+}-dependent, that modulate L-type Ca^{2+} channel gating properties.

In summary, the present data show that changes of $[Mg^{2+}]_p$ between 0.2 mM and 1.8 mM strongly suppress cardiac I_{Ca}. These data suggest that cytosolic Mg^{2+} is a potential regulator of I_{Ca} at physiological concentrations. This modulation of I_{Ca} by $[Mg^{2+}]_i$ is larger in the presence of resting levels of $[Ca^{2+}]_i$, an indication of an interaction between $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$. However, Ca^{2+}/CaM-dependent signal pathways do not appear to be involved in this modulatory action of $[Mg^{2+}]_i$. Shifts in V_m-dependent gating are also unlikely to be responsible for Mg^{2+} actions. Instead, our results suggest that the channel phosphorylation state plays a predominant role in $[Mg^{2+}]_i$-induced modulation of I_{Ca}.

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Acknowledgement

The authors wish to thank Ms Renee Green for excellent technical assistance, Drs Masato Konishi and Roman Shirokov for helpful discussions about the data. This work is supported by the National Institutes of Health (HL 69020).