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Voltage-dependent modulation of L-type calcium currents by intracellular magnesium in rat ventricular myocytes

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Abstract

Effects of changing cytosolic free Mg^{2+} concentration on L-type Ca^{2+} (I) and Ba^{2+} Ca currents (I_{Ba}) were investigated in rat ventricular myocytes voltage-clamped with pipettes containing 0.2 mM or 1.8 mM $[Mg^{2+}]_p$ ($[Mg^{2+}]_p$) buffered with 30 mM citrate acid and 10 mM ATP. Increasing $[Mg^{2+}]_p$ from 0.2 mM to 1.8 mM reduced current amplitude and accelerated its decay under a variety of experimental conditions. To investigate the mechanism for these effects, steady-state and instantaneous current-voltage relationships were studied with two-pulse and tail current (I_T) protocols, respectively. Increasing $[Mg^{2+}]_p$ shifted the V_M for half inactivation by -20 mV but dramatically decreased I_{Ca} amplitude at all potentials tested, consistent with a change in gating kinetics that decreases channel availability. This conclusion was supported by analysis of I_T amplitude, but these latter experiments also suggested that, in the millimolar concentration range, $[Mg^{2+}]$ might also inhibit permeation through open Ca^{2+}_p channels at positive V_M .

Keywords

barium current; calcium current; inactivation; tail current; voltage clamp

INTRODUCTION

Changes in cytosolic free Mg^{2+} concentration ($[Mg^{2+}]_i$) around physiologically-relevant levels have been reported to decrease peak L-type Ca^{2+} current, I_{Ca} [1-4], and to accelerate the rate of current decay [2]. These changes in I_{Ca} amplitude and decay rate can be interpreted as either an effect of $[Mg^{2+}]_i$ on Ca^{2+} permeation and/or gating kinetics of the L-type channel. In regards to the first possible mechanism, Yamaoka and Seyama [5] showed that, at physiologic levels and lower concentrations, $[Mg^{2+}]_i$ did not change single channel Ba^{2+} current amplitude in cell-attached patches. Using excised membrane patches in the presence of BayK 8644, Kuo & Hess [6] also reported that cytosolic Mg^{2+} , at concentrations greater or equal to 5 mM, could block inwardly-directed monovalent cation flux through single L-type channels, but Carbone *et al.* [7] showed that Mg^{2+} is much less able to block divalent than monovalent cation fluxes through the same channel type. These data would seem to argue against the possibility that physiologically-relevant $[Mg^{2+}]_i$ produce significant block of Ca^{2+} permeation through the L-type Ca^{2+} channel.

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Our recent data showed that $[Mg^{2+}]_i$ -dependent modulation on I_{Ca} is largely dependent on channel gating states [4]. Conditions that promoted channel opening, such as protein kinase A (PKA) phosphorylation, application of the channel agonist BayK8644 or truncation of the I_C subunit C-terminus, promoted inhibition of I_{Ca} around physiologically-relevant $[Mg^{2+}]_i$, whereas conditions lessening channel opening, such as dephosphorylation by protein phosphatase 2A or mutation of putative PKA phosphorylation sites to alanine, reduced $[Mg^{2+}]_i$ effects on I_{Ca} . These data led us to conclude that cytosolic Mg^{2+} inhibition of current is enhanced under conditions that promote channel opening.

Our finding that cytosolic Mg^{2+} inhibition of I_{Ca} is enhanced by conditions that promote channel opening raises the possibility that Mg^{2+} acts by blocking ion permeation. And, in fact such a mechanism would not be surprising given that $[Mg^{2+}]_i$, in the 1 mM range, also produces extensive block of V_M -dependent sodium and potassium channels [8,9]. For this reason, we conducted the present study to determine if cytosolic Mg^{2+} actions on I_{Ca} are attributable to block of divalent cation permeation through the L-type channel and/or changes in channel gating kinetics.

METHODS

Cell isolation

Adult rat cardiac ventricular myocytes were isolated enzymatically as described previously [3,10] from male Sprague-Dawley rats (200-225 g) 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 to 8 hours.

Measurement of membrane currents

Myocytes were placed in a chamber mounted on an inverted microscope (Nikon Inc., Japan) and superfused with a modified Tyrode's or Na^+ - and K^+ -free bathing solutions (see Solutions below). Cells were voltage clamped with electrodes containing defined concentrations of Mg^{2+} ($[Mg^{2+}]_o$) and Ca^{2+}_p using standard patch clamp techniques, as described previously [3]. K^+ currents were minimized 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's solution and, in some instances, by depolarizing the membrane potential (V_M) to -40 mV with ramp pulses prior to test protocols [3]. Tail currents, I_T , were recorded in the presence of Na^+ - and K^+ -free bathing solutions containing 0.1 μM BayK 8644.

Currents were acquired at the digitizing rates of 10 kHz and filtered at 5 kHz. Data were analyzed using pCLAMP software, version 8.0 (Axon Instruments, Union City, CA). Displayed membrane currents are shown without linear leak subtraction or capacitance correction.

Experimental protocols

Details of voltage clamp protocols are given in the text; however, current through L-type Ca^{2+} channels was calculated as a 200 M $CdCl_2$ -sensitive difference current [3]. The fraction of peak current remaining at the end of a 200-ms depolarization (r_{200}) was used to quantify the level of current decay as a function of V_M . Tail currents were also calculated as 200 M $CdCl_2$ -sensitive difference currents, and they displayed a bi-exponential decay during the test pulses. The fast component of tail current had a time constant similar to that observed for charging membrane capacitance and was therefore ignored. Current amplitude was then calculated by back-extrapolating the slow component of tail current to the beginning of the test pulse.

Drugs and Chemicals

Solutions—The pipette solution was composed of (in mM): 100 cesium gluconate, 10 PIPES (cesium salt), 15 tetraethylammonium (TEA) chloride, 0.5 NaH₂PO₄, 0.1 Tris-GTP, 5 EGTA along with ATP, Mg-ATP, citric acid, magnesium citrate, MgCl₂ and CaCl₂ to produce free [Mg²⁺] of 0.2 and 1.8 mM, pH 7.2, at specified free [Ca²⁺] of 100 nM. Total citrate was 30 mM and total ATP was 10 mM. The amounts of these reagents needed to produce specified free Mg²⁺ and Ca²⁺ concentrations were calculated using a computer program (WinMAXC 2.40, <http://stanford.edu/~cpatton/maxc.html>). For 0.2 mM [Mg²⁺], solutions included (in mM) 2.7 MgCl₂, 7.3 MgATP and 5.1 magnesium citrate, and for 1.8 mM, the amounts of these three reagents were 10, 9.6 and 15.4, respectively. Cells were superfused with a modified Tyrode's solution containing (in mM): 145 NaCl, 4 KCl, 2 (or 0.5) CaCl₂, 10 HEPES, 1 MgCl₂, and 10 glucose, pH 7.4, or a Na⁺- and K⁺-free bathing solution containing (in mM): 150 N-methyl-D-glucamine (NMG) chloride, 0.5 CaCl₂, 10 HEPES, 1 MgCl₂, and 10 glucose, pH 7.4. Experiments were performed at room temperature (22 - 25°C).

Reagents—Reagents were obtained from Sigma Chemical Corp. (St. Louis, MO), unless specified otherwise. Several reagents purchased from CalBiochem (BayK8644, 3-isobutyl-1-methylxanthine - IBMX, TTX) and forskolin (FSK, BioMol) were prepared as concentrated stock solutions that were applied to bathing solutions 30 min prior to experiments. When DMSO 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 were also prepared for control experiments. Okadaic acid (OA), from Calbiochem, was added to the electrode solution when indicated.

Data Analysis

Data are expressed as mean ± S.E.M for the number of cells indicated. Significance was determined using ANOVA and Student t-tests in commercial software (SigmaPlot, Jandel Scientific, and JMP IN, Duxbury, Pacific Grove, CA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Effect of [Mg²⁺]_i on kinetics of L-type calcium channel current

Representative current traces of *I*_{Ca} are displayed in Figure 1A (left) from cells superfused with Tyrodes solution containing 2.0 mM Ca²⁺ and voltage-clamped with patch electrodes containing salt solutions with 0.2 mM and 1.8 mM Mg²⁺. Currents were elicited by step depolarizations to 0 mV after a 1 sec ramp depolarization to -40 mV from a holding potential of -70 mV. Displayed currents were recorded 5 min after establishing the whole-cell clamp configuration, as per Wang *et al.* [3].

Increasing [Mg²⁺]_p decreased *I*_{Ca} amplitude and changed the kinetics of current decay. With 0.2 mM [Mg²⁺]_p, *I*_{Ca} displayed bi-exponential decay kinetics with a small fast component (tau = 22.4 ± 3.7 ms, n=6) and a prominent slow component (tau = 106.8 ± 13.8 ms). In contrast, *I*_{Ca} at 1.8 mM [Mg²⁺]_p displayed mono-exponential decay kinetics (tau = 60.6 ± 1.4 ms, n=5). To illustrate this point more clearly, *I*_{Ca} measured with 1.8 mM [Mg²⁺]_p was normalized to the peak current at 0.2 mM [Mg²⁺]_p. The normalized currents showed that current decay at high [Mg²⁺]_p was, initially, slower than or of a similar rate to the decay of current at low [Mg²⁺]_p. Nonetheless, after a few tens of milliseconds, current amplitude with 1.8 mM [Mg²⁺]_p continued to decline while, at low [Mg²⁺]_p, current decay slowed to a great extent (Fig. 1A, left). To evaluate the extent of current decay, the fraction of peak current remaining at the end of 200 ms depolarizations (*r*₂₀₀) was plotted as the function of *V*_M (Fig. 1A, right).

At 0.2 mM $[\text{Mg}^{2+}]_p$, r_{200} exhibited a characteristic “U” shape [11] with the a minimum value occurring at 0 mV, the same V_M where maximal I_{Ca} was observed [3]. With 1.8 mM $[\text{Mg}^{2+}]_p$, r_{200} declined monotonically with increasing depolarization and remained unchanged at positive V_M . Additionally, r_{200} for current at 1.8 mM $[\text{Mg}^{2+}]_p$ was smaller than r_{200} at 0.2 mM $[\text{Mg}^{2+}]_p$ for V_M from -10 mV to +30 mV. These results indicated the increasing $[\text{Mg}^{2+}]_i$ not only simplified the kinetics of I_{Ca} decay from a bi-exponential to a mono-exponential process but also resulted in an overall acceleration of current decay.

Our previous studies showed that regulation of L-type Ca^{2+} channels by $[\text{Mg}^{2+}]_i$ is prominent under the conditions that increase channel opening probability [4]. Therefore, we investigated how $[\text{Mg}^{2+}]_i$ affected the L-type Ca^{2+} current decay under these experimental conditions. Channel opening probability was increased under channel phosphorylation conditions (60 minute preincubation with IBMX and FSK; inclusion of okadaic acid in the patch electrode solution) or by application of BayK8644, coupled with PP_{2A} , as previously published [4]. Under high channel phosphorylation conditions, current decay exhibited bi-exponential kinetics at 0.2 mM $[\text{Mg}^{2+}]_p$. The fast component of current, more prominent than under basal conditions (Fig. 1B, left), is thought to be related to Ca^{2+} -dependent inactivation of I_{Ca} , which depends on Ca^{2+} influx via the Ca^{2+} channels [12], and Ca^{2+} release from the sarcoplasmic reticulum [13-17]. Analogous to control conditions, increasing $[\text{Mg}^{2+}]_p$ from 0.2 to 1.8 mM changed the current decay from a bi-exponential to a mono-exponential process and, in general, accelerated the current decay (Fig. 1B, left). Moreover, the rate of current decay with 1.8 mM $[\text{Mg}^{2+}]_i$ was slower than the fast component of current decay at 0.2 mM $[\text{Mg}^{2+}]_p$. Similarly, under conditions promoting low phosphorylation but high channel opening, induced by including PP_{2A} (1 unit/ml) in the patch electrode solution and 0.1 M BayK8644 in the superfusion solution, the same increase in $[\text{Mg}^{2+}]_p$ simplified the current decay from a bi-exponential to a mono-exponential process and produced acceleration of current decay (Fig. 1B, right). As in Fig. 1A, values of r_{200} were also decreased in the presence of the higher $[\text{Mg}^{2+}]_p$ under conditions promoting greater channel opening (not shown). Thus, under all conditions tested, increasing $[\text{Mg}^{2+}]_i$ affected kinetics of current decay in a qualitatively similar manner, accelerating current decay while simplifying that decay to an exponential process.

Changes in the kinetics of current decay might imply an alteration in Ca^{2+} channel inactivation. L-type Ca^{2+} channel inactivation is thought to be mediated via the rapid Ca^{2+} -dependent process and a relatively slower V_M -dependent process [11,18]. V_M -dependent current inactivation is readily observed with minimal Ca^{2+} -dependent inactivation by using Ba^{2+} as the permeating ion [19]. Thus the effects of $[\text{Mg}^{2+}]_p$ on current decay were examined with Ba^{2+} as the permeant ion. As expected, Ba^{2+} current through L-type channels displayed a slow and incomplete decay with 0.2 mM $[\text{Mg}^{2+}]_p$, presumably a reflection of the time course of V_M -dependent inactivation. Interestingly, increasing $[\text{Mg}^{2+}]_p$ from 0.2 to 1.8 mM caused Ba^{2+} current to show much more rapid and complete decay (Fig. 1C, left), consistent with the results of Hartzell & White [2]. With 1.8 mM $[\text{Mg}^{2+}]_p$, Ba^{2+} current decayed in a mono-exponential fashion with a time constant at 0 mV of 45.8 ± 1.8 ms ($n=6$), similar to the time constant for decay of Ca^{2+} current measured with this $[\text{Mg}^{2+}]_p$. The value of r_{200} declined monotonically with increasing depolarization at both low and high $[\text{Mg}^{2+}]_p$ but r_{200} values were much smaller at 1.8 mM $[\text{Mg}^{2+}]_p$ over the V_M range tested. In summary, the effects of increasing $[\text{Mg}^{2+}]$ on Ba^{2+} currents were qualitatively similar to those effects on Ca^{2+}_p currents, i.e. greatly decreasing current amplitude and accelerating current decay kinetics.

Taken together, the results above show that increasing $[\text{Mg}^{2+}]_p$ simplified and accelerated the kinetics of current decay, irrespective of the conditions under which L-type Ca^{2+} channel current was measured. As presented in the Discussion, the simplest but not the only explanation

for these results is that Mg^{2+} alters current amplitude and decay rate by acting as an open channel blocker, as has been previously shown for K^+ channels [9].

Effect of $[\text{Mg}^{2+}]_i$ on V_M dependence of I_{Ca} steady-state inactivation

Is there any evidence that cytosolic Mg^{2+} modulation of L-type Ca^{2+} channels might not depend on block of ion permeation? To address this issue, the effect of $[\text{Mg}^{2+}]_p$ on steady state current inactivation was investigated, since this channel property depends on closed and inactivated, i.e. nonconducting, channels. To study steady state inactivation, a two pulse protocol was used in which a prepulse between -90 and 0 mV (in 10 mV increments) was elicited prior to a 200 msec test depolarization to 0 mV (Fig. 2, top). Preliminary experiments (not shown) indicated that recovery from inactivation required approximately 13 seconds. For this reason, the duration of the prepulse was set to 20 sec to insure that channels had reached steady state prior to the test depolarization.

Representative current tracings at prepulse of -30 mV with low and high $[\text{Mg}^{2+}]_p$ are displayed in the middle panel of Figure 2. Current amplitude during the prepulse was initially similar. With 0.2 mM $[\text{Mg}^{2+}]_p$, current was essentially unchanged during the 20 sec prepulse but, with 1.8 mM $[\text{Mg}^{2+}]_p$, current decayed very slowly. Consequently, during the test depolarization to 0 mV, I_{Ca} was greatly reduced with 1.8 mM $[\text{Mg}^{2+}]_p$. The currents induced by the test depolarization are plotted as the function of the prepulse potential (Fig. 2, bottom). The potentials for half-maximal inactivation of I_{Ca} with 0.2 mM and 1.8 mM $[\text{Mg}^{2+}]_p$ were -31.4 ± 2.4 mV ($n=4$) and -51.5 ± 1.3 mV ($n=6$), respectively, i.e. increasing $[\text{Mg}^{2+}]_p$ shifted inactivation to more negative V_M by -20.1 ± 1.3 mV ($n=4$). These results suggested that $[\text{Mg}^{2+}]_p$ can affect the V_M -dependence of channel inactivation. Perhaps a more interesting result is that I_{Ca} amplitude with high $[\text{Mg}^{2+}]_p$ was much smaller than with low $[\text{Mg}^{2+}]_p$ at all V_M . Even at the negative V_M range where the current during the test depolarization was not affected by prepulse potential, increasing $[\text{Mg}^{2+}]_p$ from 0.2 mM to 1.8 mM decreased I_{Ca} amplitude by approximately 60%. Thus, in addition to any effects on the V_M dependence of channel gating, $[\text{Mg}^{2+}]_i$ reduced I in a V_M -independent manner that suggested $[\text{Mg}^{2+}]_i$ might affect Ca^{2+} Ca channel availability, in other words, channel gating.

Effects of $[\text{Mg}^{2+}]_i$ on voltage dependence of L-type Ca^{2+} tail currents

Our conclusion that cytosolic Mg^{2+} affects L-type Ca^{2+} channel availability could be complicated if cytosolic Mg^{2+} can block ion permeation through open Ca^{2+} channels during the test depolarization to 0 mV (see Discussion). Whether and to what extent Mg^{2+} blocks ion permeation through the open channel at this V_M is unknown. However, our published data [4] and data in Fig. 1 may be consistent with cytosolic Mg^{2+} acting as an open channel blocker. Thus, the possibility that cytosolic Mg^{2+} , at the concentrations used, might block open Ca^{2+} channels was evaluated by comparing L-type Ca^{2+} channel tail currents (I_T) with 0.2 and 1.8 mM $[\text{Mg}^{2+}]_p$. Cells were first depolarized to +10 mV for 10 ms to maximally open Ca^{2+} channels with a minimum of inactivation. The membrane was then stepped immediately to test potentials ranging from -80 mV to +30 mV for 50 ms to elicit I_T (Fig. 3A). Figure 3B shows representative current tracings at a test potential of -20 mV for I_T from ventricular myocytes at 0.2 mM and 1.8 mM $[\text{Mg}^{2+}]_p$. Currents were recorded during superfusion with a Na^+ - and K^+ -free (substituted with equimolar NMG) Tyrodes solution containing 0.1 μM BayK8644. As expected, I_T was considerably smaller in the cell voltage-clamped with 1.8 mM $[\text{Mg}^{2+}]_p$.

Figure 3C shows that, as the test potential was depolarized from -80 mV to +30 mV, I_T decreased in amplitude. The curvilinear relationship between current amplitude and test potential suggests that I_T behaves as predicted by the constant field equation [20], as has been

observed previously for Ca^{2+} currents reviewed in Hagiwara & Byerly [21]. In addition, I_T was decreased at 1.8 mM $[\text{Mg}^{2+}]_p$ for the all test potentials.

Instantaneous I_T amplitude in any condition reflects the permeability properties of the open channel under investigation, independent of channel gating properties. However, in comparing two different experimental conditions, the *a priori* assumption that the number of open channels is the same cannot be made. Nonetheless, even if the absolute number of open Ca^{2+} channels changes with $[\text{Mg}^{2+}]_p$, the relative number of channels should be constant. Therefore, if cytosolic Mg^{2+} has no effect of channel permeability properties, the ratio of I_T should be constant at all V_M , but the ratio should change if ion permeability is affected by Mg^{2+} (see Discussion).

To investigate this point, tail current amplitude measured with 1.8 mM $[\text{Mg}^{2+}]_p$ was divided by that at 0.2 mM $[\text{Mg}^{2+}]_p$ and plotted as the function of V_M (Fig. 3D). The ratio of I_T was not V_M -dependent with V_M from -80 mV to -20 mV, but at more positive V_M , the ratio decreased progressively. These results suggest that increasing $[\text{Mg}^{2+}]_p$ from 0.2 to 1.8 mM does affect the permeability properties of the open Ca^{2+} channel, but only at V_M approaching or exceeding 0 mV. Over a broad range of negative V_M , this change in $[\text{Mg}^{2+}]_p$ had no effect on the ratio of I_T and, therefore, did not appear to affect ion permeability properties of the channel.

Interestingly, the maximal ratio of I_T observed at these negative V_M , approximately 0.6, was significantly less than 1, a clear demonstration that increasing $[\text{Mg}^{2+}]_p$ inhibited Ca^{2+} channel opening independent of V_M . This V_M -independent effect on I_T cannot reflect open channel permeability properties and therefore must reflect a change in the number of channels that are open at the different $[\text{Mg}^{2+}]_p$ (see Discussion). In other words, increasing $[\text{Mg}^{2+}]_i$ decreased Ca^{2+} channel availability. These results also suggest that the decrease in I_{Ca} observed in Fig. 2 reflects, for the most part, a true reduction in Ca^{2+} channel availability with increased $[\text{Mg}^{2+}]_p$.

Another observation in these experiments was the effect of increasing $[\text{Mg}^{2+}]_i$ on the decay kinetics of the tail currents. Figure 3E shows that the time constant for tail current decay was unchanged at those negative V_M where ratio of tail currents was constant; however, at more positive V_M , increasing $[\text{Mg}^{2+}]_i$ accelerated current decay in addition to reducing the tail current ratio.

DISCUSSION

The most prominent effect on L-type Ca^{2+} current of changing $[\text{Mg}^{2+}]_i$ around its physiological concentration range is a marked modulation of current amplitude shown here and in previous papers [1-5,22,23]. This modulation in current amplitude might be explained by the change in channel gating and/or permeation rate through the ion channels. The motivation for the present study was to establish whether $[\text{Mg}^{2+}]_i$, under the conditions of our experiments, was having a prominent effect on ion permeation rate.

The results of previous studies suggest that the concentrations of cytosolic Mg^{2+} used in our experiments would not likely block Ca^{2+} permeation through the L-type Ca^{2+} channel pore. Yamaoka and Seyama [5] measured single channel Ba^{2+} currents with cell-attached patch electrodes while whole-cell voltage-clamping cardiac myocytes with patch-electrodes containing micromolar Mg^{2+} concentrations. During the time course of the experiment, in which $[\text{Mg}^{2+}]_i$ presumably declined towards the micromolar level, single channel Ba^{2+} current amplitude did not change. These results suggested that $[\text{Mg}^{2+}]_i$ up to those approaching physiologic levels did not block Ba^{2+} permeation. One caveat in extrapolating these results to the present data or other published whole cell current data is the high concentration of Ba^{2+} used in the cell-attached patch electrode solution (90 mM). This high concentration of permeant

cation might complicate comparisons to whole cell current recordings where the permeant ion concentration is in the neighborhood of 1 millimolar in the extracellular solution.

Kuo & Hess [6] also studied the effect of Mg^{2+} on ion permeation through L-type Ca^{2+} channels using excised inside-out membrane patches exposed to the Ca^{2+} channel agonist, Bay K8644. Their studies showed that Mg^{2+} on the intracellular side of the membrane could block ion permeation at high (micromolar) and low affinity sites. However, with inwardly directed currents, Mg^{2+} did not appear to block the channel at the high affinity sites unless the current-carrying ion was Li^+ , which interacts with the channel at low affinity [24]. Mg^{2+} interacting with the channel at its low affinity sites was observed to decrease single channel Li^+ current amplitude, but the concentration that produced a half-maximal decrease in permeation rate was calculated to be 10 mM at 0 mV [6]. The low affinity Mg^{2+} site also appeared to be relatively non-selective for cations, so their estimated value of Mg^{2+} affinity might be relevant to the situation where Ca^{2+} is the permeating ion. In any case, the affinity of Mg^{2+} for the low affinity site in the L-type channel permeation pathway would seem to indicate that the highest $[Mg^{2+}]_i$ used in the present study, 1.8 mM, would not have a significant effect on ion permeation. Nonetheless, given the assumptions and extrapolations needed to arrive at this conclusion, it is important to experimentally test whether $[Mg^{2+}]_i$ could be blocking ion permeation under the conditions of our experiments [3,4], that are similar to those of other reports in which $[Mg^{2+}]_i$ has been reported to decrease whole-cell Ca^{2+} currents [1,5,22,23, 25].

Are there data suggesting that $[Mg^{2+}]_i$ used here could block ion permeation? Wang & Berlin [4] studied that effect of $[Mg^{2+}]_i$ on wild-type and mutant L-type Ca^{2+} channels and came to the conclusion that experimental maneuvers producing an increase in channel opening also promoted the ability of cytosolic Mg^{2+} to inhibit current. This finding could be consistent with Mg^{2+} acting as an open channel blocker. The findings of White & Hartzell [1] and Pelzer *et al.* [25] that channel phosphorylation promotes the reduction of Ca^{2+} current by cytosolic Mg^{2+} current might also be consistent with Mg^{2+} block of ion permeation through the open channel. This possibility motivated the present experiments to determine if $[Mg^{2+}]_i$ used here could decrease current in a manner consistent with permeation block.

We used instantaneous I-V relationships to investigate whether $[Mg^{2+}]_i$ could be acting as an open channel blocker. Instantaneous I-V relationships, measured from the peak amplitude of tail currents, provide information on the permeation properties of an ion channel, independent of time-dependent changes in gating. The results showed that tail currents through Ca^{2+} channels display a curvilinear dependence on test potential at both $[Mg^{2+}]_p$ tested, consistent with the predictions of the constant field equation [21]. Taking the ratio of tail current amplitudes measured at each V_M allowed us to look at relative changes in ion permeation through open Ca^{2+} channels with 0.2 and 1.8 mM $[Mg^{2+}]_p$. However, in order to interpret the tail current ratio, it is necessary to assume that Mg^{2+} block of ion permeation in an open channel would occur at a site in the membrane electric field. This assumption is based on the results of Kuo & Hess (1993) showing that low-affinity intracellular Mg^{2+} block occurs at a site located 30% of the way through the membrane electric field from the inside. Then, using eqns. 1 and 2 from Kuo & Hess [6], it is possible to predict the relative degree of Ca^{2+} channel current at two different $[Mg^{2+}]_i$, $[Mg^{2+}]_a$ and $[Mg^{2+}]_b$, as a function of V_M :

$$\text{Current Ratio} = \frac{[Mg^{2+}]_a + K_D^0 * \exp(-2\Theta F V_M / RT)}{[Mg^{2+}]_b + K_D^0 * \exp(-2\Theta F V_M / RT)}$$

where K_D^0 is the Mg^{2+} binding affinity for the blocking site in the permeation pathway at 0 mV, Θ is a proportionality factor and F , R and T have their usual meanings. This equation predicts that, at negative V_M , the current ratio will asymptotically approach a value of 1, i.e.

no significant block of ion permeation occurs, but the ratio will approach the value of $[Mg^{2+}]_a/[Mg^{2+}]_b$ at positive V_M , in this instance a value of 0.11.

We observed that the ratio of tail currents was constant at a value of approximately 0.6 for negative V_M , but progressively decreased at V_M approaching and exceeding 0 mV. Thus, the data clearly do not fit with the predictions for a purely V_M -dependent permeation blocker. Instead, two mechanisms, one V_M -independent and the other V_M -dependent, are necessary to explain the properties of the current ratio. Given the design of the protocol, in which peak tail current amplitude is determined by the number of open channels and the channel conductance, we can conclude that the V_M -independent reduction of current ratio must reflect a decrease in the number of channels open in the presence of 1.8 mM $[Mg^{2+}]$, i.e. Ca^{2+}_p channel availability is decreased, as seen in Fig. 2, is presumably due to altered gating kinetics.

Given this outcome, our experiments suggest that the predominant mechanism of current inhibition on increasing $[Mg^{2+}]_i$ from 0.2 to 1.8 mM is a decrease in channel availability, except at V_M equal or exceeding +30 mV. Thus, block of ion permeation would not seem to be the major mechanism underlying the reduction of Ca^{2+} current amplitude, and it probably plays a less prominent role in reducing current amplitude under the conditions of experiments in Fig. 1.

While the most prominent effect of changing $[Mg^{2+}]_i$, in its physiological concentration range, is suppression of current amplitude [1-4], acceleration of current decay is also notable, as shown by the reduction of τ_{200} with increased $[Mg^{2+}]_i$. Hartzell & White [2] also observed similar effects of increased $[Mg^{2+}]_i$ on the decay of Ca^{2+} current. The mechanism explaining this acceleration of current decay was not explored thoroughly in the present experiments. Nonetheless, it seems possible that permeation block by cytosolic Mg^{2+} might play a role in this phenomenon. The basis for this conclusion also comes from the tail current experiments where decay rate was accelerated at higher $[Mg^{2+}]_i$ only at V_M positive to -20 mV (Fig. 3E), where the ratio of tail currents was also decreasing and we observe V_M -dependent effects on current ratio that we attribute to permeation effects of cytosolic Mg^{2+} . Currents in Fig. 1 were measured at 0 mV. Consequently, the acceleration of current decay in these experiments might be due, at least in part, to Mg^{2+} block of ion permeation.

Cytosolic Mg^{2+} effects on ion permeation are unlikely to fully explain the acceleration of current decay. Increasing $[Mg^{2+}]_i$ changed the kinetics of decay from a bi-exponential to a single exponential process where the decay time constant in the presence of 1.8 mM $[Mg^{2+}]_p$ was slower than the fast component, but faster than the slow component, of current decay observed with 0.2 mM $[Mg^{2+}]_p$. This effect of $[Mg^{2+}]_i$ was observed under a wide variety of conditions including the presence of BayK8644, high phosphorylation conditions induced by protein kinase A, and Ba^{2+} as the permeating divalent cation. These changes in current kinetics were also not due to a simple shift in the V_M dependence of current inactivation. The mechanism underlying the observed changes in current decay kinetics will require further investigation.

Finally, a -20 mV shift in the V_M dependence of steady state current inactivation was observed on increasing $[Mg^{2+}]$. Previously, we have reported a 5-10 mV shift of Ca^{2+}_p current inactivation in the negative direction with shorter conditioning pulses (up to 3 sec in duration), and a similar shift has been observed by others [2]. This smaller shift has been attributed to screening of membrane surface charges. We have only observed the larger 20 mV shift with the longer prepulses necessary to achieve true steady state inactivation and these results suggest that cytosolic Mg^{2+} may have a greater effect on some channel closed states, consistent with its ability to decrease channel availability. This point, however, also requires further investigation.

In summary, the reduction of Ca^{2+} current amplitude produced by increasing $[\text{Mg}^{2+}]_i$ across its physiologic range appears to depend predominantly on a decrease in channel availability rather than block of ion permeation. Once the channel opens, it is possible that cytosolic Mg^{2+} has the opportunity to block ion permeation, especially at those membrane potentials encountered during physiologic processes such as the plateau of a cardiac ventricular action potential.

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A **B**

C

Figure 1.

Effect of $[Mg^{2+}]_p$ on L-type Ca^{2+} channel currents. **A**, I_{Ca} recorded during depolarizations to 0 mV in rat ventricular myocytes voltage-clamped with patch electrodes containing (1) 0.2 mM $[Mg^{2+}]_p$ and (2) 1.8 mM $[Mg^{2+}]_p$. Trace (3) is I_{Ca} with 1.8 mM $[Mg^{2+}]_p$ normalized to the amplitude of I_{Ca} with 0.2 mM $[Mg^{2+}]_p$ (*left*). The fraction of peak I_{Ca} remaining at the end of 200-ms depolarizations (r_{200}) with 0.2 mM (●, $n=6$) and 1.8 mM $[Mg^{2+}]_p$ (▲, $n=5$) at different test potentials (V_M) (*right*). Significant changes of r_{200} between 0.2 mM $[Mg^{2+}]_p$ and 1.8 mM $[Mg^{2+}]_p$ are indicated by asterisks. **B**, tracings of I_{Ca} , as in part A, except with the addition of the indicated compounds. **C**, I_{Ba} recorded during depolarizations to 0 mV. Current traces (1) - (3) are indicated as in Part A (*left*); r_{200} for I_{Ba} was measured with 0.2 mM (●, $n=7$) and 1.8 mM $[Mg^{2+}]_p$ (▲, $n=6$) and plotted versus test potential (*right*).

Figure 2.
Effect of $[Mg^{2+}]_p$ on the V_M dependence of steady state I_{Ca} inactivation. Two-pulse inactivation protocol with a 20 sec prepulse and 200 msec test depolarization to 0 mV (*top*). Typical currents with a prepulse of -30 mV where traces (1) and (2) were recorded with 0.2 and 1.8 mM $[Mg^{2+}]_p$, respectively (*middle*). I_{Ca} plotted versus prepulse potential (V_M) for myocytes voltage-clamped at 0.2 (●, n=6) and 1.8 mM $[Mg^{2+}]_p$ (▲, n=6) (*bottom*).

Figure 3.

Effect of $[Mg^{2+}]_p$ on L-type calcium channel tail currents (I_T). **A**, voltage protocol used to measure instantaneous I_T - V_M relationships. **B**, sample records of I_T in cardiac myocytes dialyzed with 0.2 mM (1) and 1.8 mM $[Mg^{2+}]_p$ (2) at a test potential of -20 mV. I_T was recorded in the presence of 0.1 μ M BayK8644, 5 min after establishing the whole-cell patch-clamp. **C**, instantaneous I_T - V_M relationships in cells dialyzed with 0.2 mM ($\bullet$, $n=5$) and 1.8 mM $[Mg^{2+}]_p$ ($\blacktriangle$, $n=5$). **D**, the ratio of I_T , defined as peak I_T recorded with 1.8 mM $[Mg^{2+}]_p$ at one V_M divided by that with 0.2 mM $[Mg^{2+}]_p$, is plotted against V_M ($n=5$). The dashed curve was fit to the data with an arbitrary function. **E**, time constant of I_T decay (τ) plotted against V_M for myocytes dialyzed with 0.2 mM ($\bullet$, $n=5$) and 1.8 mM $[Mg^{2+}]_p$ ($\blacktriangle$, $n=5$). Time constants

were determined by fitting the change in current during the 50 msec test pulse at each V_M without accounting for current levels observed in the presence of 200 M CdCl_2 . Asterisks indicate significant changes of τ between 0.2 and 1.8 mM $[\text{Mg}^{2+}]_p$.