Temperature-sensitive intracellular Mg$^{2+}$ block of L-type Ca$^{2+}$ channels in cardiac myocytes

KAORU YAMAOKA, TSUNETSUGU YUKI, KAYOKO KAWASE, MAKOTO MUNEMORI, AND ISSEI SEYAMA
Department of Physiology, School of Medicine, Hiroshima University, Minami-Ku, Hiroshima 734-8551, Japan
Received 5 July 2001; accepted in final form 6 November 2001

Mg$^{2+}$ is abundant in the cytoplasm and exerts several regulatory functions in cardiac myocytes. Intracellular Mg$^{2+}$ (Mg$_{i}$) acts as a stimulator, often with ATP, whether it is phosphorylation dependent or not (18, 32). Blocking effects of Mg$_{i}$ have been also reported (1, 26–29). Pelzer et al. (20) presented both stimulatory and inhibitory effects of Mg$_{i}$ on the L-type Ca$^{2+}$ channel in guinea pig ventricular myocytes. How are these Mg$_{i}$ effects physiologically relevant? As to the Mg$^{2+}$ block, we have previously postulated that cAMP-dependent phosphorylation reduces the sensitivity to Mg$^{2+}$ block so that the L-type Ca$^{2+}$ channel can be activated by β-adrenoreceptor stimulation in the heart (29). However, similar blocking effects of Mg$^{2+}$ for mammalian cardiac myocytes have been lacking with the exception of a single brief report (1). Moreover, Hirahara et al. (9) reported that intracellular dialysis with Mg$^{2+}$-depleting solutions produced only a transient and marginal increase of L-type Ca$^{2+}$ channel current (I$_{Ca}$) in guinea pig ventricular myocytes. Recently, Pelzer et al. (20) have shown a modest Mg$^{2+}$ blocking effect that disappear in the presence of the nonspecific protein kinase inhibitor K252a. In our hands, the Mg$^{2+}$ block persisted even under the condition that the phosphorylation process was completely inhibited by the depletion intracellular ATP (28). The question arose as to whether the same Mg$^{2+}$-dependent block observed in frog cardiomyocytes exists in the mammalian heart. According to our hypothesis that phosphorylation of the L-type Ca$^{2+}$ channel in frog ventricular myocytes increases I$_{Ca}$ by relieving Mg$^{2+}$-dependent block of the channel, a similar mechanism may operate in guinea pig ventricular myocytes, because I$_{Ca}$ of guinea pig ventricular myocytes is regulated by cAMP-dependent phosphorylation (19, 25).

In this study, we employed a method of intracellular dialysis to vary the concentration of Mg$^{2+}$ inside of guinea pig ventricular myocytes. We found that temperature is a key factor controlling the Mg$^{2+}$-dependent regulation of the L-type Ca$^{2+}$ channel in guinea pig as well as frog ventricular myocytes.

MATERIALS AND METHODS

Cell preparation. Single ventricular cells were obtained from either guinea pig or frog hearts by enzymatic dissociation as previously described (8, 22). Animals were used in accordance with the “Guiding Principles for the Care and Use of Animals” approved by the Council of the Physiological Society of Japan. Adult male guinea pigs weighing 200–400 g were anesthetized with pentobarbitone sodium (30 mg/kg ip), and, under artificial respiration, the heart was rapidly removed. Frogs were killed by decapitation, the spinal cord was destroyed, and the heart was then excised. The heart of either species was mounted on a Langendorff apparatus for retrograde perfusion of the aorta. Hearts were perfused (15–20 min at 32°C) with Ca$^{2+}$-free Tyrode solution (guinea pig) or Ca$^{2+}$-free Ringer solution (frog) (see Solutions and chem-
calcium) supplemented with collagenase (0.12 mg/ml) (guinea pig) or with a mixture of Yakult collagenase (0.04 mg/ml, Yakult), Wako collagenase (0.40 mg/ml, Wako Pure Chemical Industries), and type III trypsin (0.06 mg/ml, Sigma) (frog). The hearts were then rinsed with storage solution (see Solutions and chemicals), and cells were dispersed and filtered through a nylon mesh (200 μm). Dispersed cells were collected by centrifugation at 65 g for 1 min, maintained at room temperature for the first hour, and finally stored at 4°C until used in experiments.

Solutions and chemicals. Tyrode solution was used as the standard external solution for guinea pig ventricular myocytes. Ca\(^{2+}\)-free Tyrode solution contained (in mM) 135 NaCl, 1.0 MgCl\(_2\)-6H\(_2\)O, 5.4 KCl, 0.33 NaH\(_2\)PO\(_4\)-2H\(_2\)O, 10.0 HEPES, and 5.5 glucose; pH 7.4 (adjusted with NaOH). To record \(I_{\text{Ca}}\) in guinea pig myocytes, Na\(^+-\)free external and internal solutions were used. Na\(^+-\)free external solution was composed of (in mM) 137 N-methyl-d-glucamine (NMDG)-HCl, 20 CsCl, 5 glucose, 10 HEPES, and 2 CaCl\(_2\); pH 7.4 (adjusted with CsOH). In the experiments shown in Fig. 6C, the external solution for Na\(^+-\)free external solution was replaced with NaCl and TTX (3 μM) was added (100% Na\(^+-\)free external solution). Na\(^+-\)free internal solution was composed of (in mM) 136 CsCl, 3.0 tris(hydroxymethyl)aminomethane ATP salt (Tris-ATP), 10.2 bis(2-amino-phenoxo)-ethane-N,N,N’,N’-tetraacetic acid (BAPTA), 5 EDTA, and 10 HEPES; pH 7.2 (adjusted with CsOH).

The standard external solution used for the recording of \(I_{\text{Ca}}\) in frog myocytes contained (in mM) 113.5 NaCl, 5.4 CsCl, 2.0 CaCl\(_2\), and 5.0 HEPES; this solution was supplemented with 0.3 μM TTX (Sankyo). Ca\(^{2+}\)-free solution for the frog myocytes contained (in mM) 93.5 NaCl, 5.4 KCl, 5.0 MgSO\(_4\), 20.0 glucose, 20.0 taurine, and 10.0 HEPES. The pH of all external solutions for frog myocytes was adjusted to 7.2 with NaOH. The standard internal solution for frog myocytes consisted of (in mM) 100 CsCl, 3.0 disodium creatine phosphate, 3.0 Tris-ATP, 10.0 BAPTA, 5.0 EDTA, and 10 HEPES; pH 7.2 (adjusted with CsOH).

In changing Mg\(^{2+}\)-concentration (Mg\(^{2+}\)\(_{\text{in}}\)), total concentrations of MgCl\(_2\) and CaCl\(_2\), calculated according to Schoenmakers et al. (21), were added to the internal solutions to obtain desirable free [Mg\(^{2+}\)] at pCa = 8 and pH = 7.0 for the frog and pH = 7.2 for the guinea pig at given temperatures. The program automatically calculates metal-chelator stability constants for effects of ionic strength, temperature, and pH.

KB medium (12) was used as a storage solution for guinea pig myocytes and contained (in mM) 50.0 l-glutamic acid, 40.0 KCl, 20.0 KH\(_2\)PO\(_4\), 20.0 taurine, 3.0 MgCl\(_2\), 10.0 glucose, and 10.0 HEPES; pH 7.4 (adjusted with KOH). For frog myocytes, modified KB medium composed of (in mM) 70 KCl, 20 K\(_2\)HPO\(_4\), 5.0 MgSO\(_4\), 5.0 pyruvate, 20 taurine, 5.0 creatine, 5.0 succinate, 0.1 K\(_2\)ATP, 10 glucose, and 0.04 EGTA (pH 7.0, adjusted with KOH) was used.

GTP was purchased from Yamasa. Tris-ATP, BAPTA, EDTA, disodium creatine phosphate, and forskolin (FSK) were purchased from Sigma. Unless otherwise stated, all other chemicals were purchased from Wako Pure Chemical Industries.

Temperature control. The temperature of the bath liquid was measured using a platinum resistance thermometer (dimensions, 2.3 \(\times\) 2.3 \(\times\) 2.0 mm, type RMB11101, Yamari Industries). Temperature was manually controlled using a Pelletier device (NetsuDenShi Kogyo) that heated or cooled (by means of direct feedback current) the polyethylene tubing (outer diameter, 1.5 mm; length, 10 cm) carrying liquid to the experimental chamber. At the same time, excess heat was drawn from the Pelletier block by coolant water circulated through an attached brass fixture (10 ml in volume). The flow rate of the external perfusate was kept constant at 1 ml/min so that total replacement of the solution in the chamber (200-μl volume) was complete within 2 min. Bath temperature could be well controlled between 9 and 40°C at an ambient temperature of \(-24°C\).

Electrophysiological recording and data analysis. Whole cell Ca\(^{2+}\) currents (\(I_{\text{Ca}}\)) for both the guinea pig and frog ventricular myocytes were recorded using a patch-clamp amplifier (Axopatch 200A, Axon Instruments) and filtered through a four-pole Bessel low-pass filter with a cutoff frequency of 5 kHz. Curve fits and data analysis were performed with pCLAMP software (Axon Instruments). Pipette resistance was 1–1.5 MΩ when filled with Na\(^+-\)-free internal solution. Membrane capacitance was determined from the current amplitude elicited in response to hyperpolarizing (from a holding potential of \(-80\) to \(-130\) mV for 50 ms) and depolarizing (from \(-130\) to \(-80\) mV for 50 ms) ramp voltage pulses at a rate of 1.0 V/s. Capacitance measurements by the ramp pulses were always made at the beginning and end of each experiment. The averaged cell capacitances used in this study were 74.0 ± 1.6 pF (n = 134) for guinea pig ventricular myocytes and 77.8 ± 5.2 pF (n = 30) for frog ventricular myocytes. In addition, cell capacitance compensation in combination with the series resistance (\(R_s\)) compensation (70–80%) of the Axopatch 200A amplifier was used to monitor cell capacitance and \(R_s\) throughout the experiments. Separately, in a series of experiments (see Fig. 6), cell capacitance and \(R_s\) (5.26 ± 0.88 MΩ, n = 31) were monitored but without using \(R_s\) compensation after each protocol of obtaining current-voltage relationships (see below) by applying a voltage ramp as indicated above together with a 20-mV hyperpolarizing voltage step from a holding potential of \(-80\) mV. This enabled us to estimate efficiency of cell dialysis. \(R_s\) measured with voltage steps were comparable with \(R_s\) readings of the Axopatch 200A amplifier and were usually twice the pipette resistance. Cells were directly transferred from KB medium to the experimental chamber, and current recordings were started after 5- to 10-min perfusions of the external solutions. Approximately 60–70% of guinea pig cells subjected to Na\(^+-\)-free external solution were quiescent. Currents other than \(I_{\text{Ca}}\) were eliminated almost totally by the use of the internal and external solutions specified above. \(I_{\text{Ca}}\) was monitored every 5 s with clamp steps from -40 to +40 mV in 10-mV increments from a holding potential of \(-80\) mV. For guinea pig myocytes, to inactivate outward-going current through TTX-sensitive Na\(^+\) channels and inward-going current through T-type Ca\(^{2+}\) channels, a 200-ms conditioning prepulse to \(-40\) mV was applied just before eliciting \(I_{\text{Ca}}\). \(I_{\text{Ca}}\) amplitude was determined as the absolute value of the peak inward current, because leakage currents were usually negligible (otherwise the data were excluded). In guinea pig and frog ventricular cells, peak \(I_{\text{Ca}}\) was always observed at transmembrane potentials between 0 and \(-20\) mV with the 10-mV step pulse protocol described above.

Data are expressed as means ± SE. Statistical significance between groups was determined by unpaired two-tailed Student's t-test, with P < 0.05 considered statistically significant.

RESULTS

** Pronounced temperature sensitivity of \(I_{\text{Ca}}\) increased by low-Mg\(^{2+}\)-solutions. A sudden change in temperature from 24 to 32°C caused an increase in the amplitude of \(I_{\text{Ca}}\) and a shortening of time to peak and...**
inactivation time constants ($\tau_{\text{inact}}$) (Fig. 1; see Fig. 8 for quantitative comparison). The effect of raised temperature was reversible at an intracellular pMg of 3.0 (Fig. 1A). Although a significant rundown was detected after the first temperature change, the second application of heating exhibited the same reversible response to temperature. Temperature change in the same range between 24 to 32°C produced a much more pronounced effect on the amplitude of $I_{\text{Ca}}$ (Fig. 1B) when cells were dialyzed with a low-Mg$^{2+}$ solution (pMg of 6) (Fig. 1B). In this case, intermediate temperature at 28°C could produce a gradual increase in $I_{\text{Ca}}$ at an pMg of 6. Nonetheless, the effect of elevated temperature under these conditions was not reversible, i.e., returning the temperature from 32 to 24°C did not restore $I_{\text{Ca}}$ to the original level at 24°C. This indicates that the enhancing effect of the low-Mg$^{2+}$ solution (pMg = 6) on $I_{\text{Ca}}$, which has been shown previously in frog ventricular myocytes (28), can only be seen at temperatures higher than 24°C in guinea pig ventricular myocytes. In frog ventricular myocytes, the enhancing effect of low-Mg$^{2+}$ solutions was suppressed in the presence of intracellular GTP (0.4 mM) (27). The same was true in guinea pig ventricular myocytes. Addition of 0.4 mM GTP to the low-Mg$^{2+}$ (pMg = 6) solution suppressed the potentiation of $I_{\text{Ca}}$ induced by low Mg$^{2+}$ (Fig. 2). There was a statistically significant effect ($P < 0.01$) of GTP when $I_{\text{Ca}}$ was compared (asterisk in Fig. 2; 15.3 ± 0.6 pA/pF with GTP, $n = 4$, and 41.2 ± 4.0 pA/pF without GTP, $n = 5$). Thus the potentiation of $I_{\text{Ca}}$ induced by low Mg$^{2+}$ is qualitatively the same in guinea pig ventricular myocytes at 32°C and frog ventricular myocytes at 24°C.

**Fig. 1.** Influence of bath temperature and pipette free Mg$^{2+}$ concentration on current through L-type calcium channels ($I_{\text{Ca}}$) of guinea pig ventricular myocytes. A and B: representative time course for peak $I_{\text{Ca}}$ during step changes of bath temperature between 24 and 32°C (left). Current recordings at the indicated times (a–d) are shown along with the pulse protocol used (right). (Fast Na$^+$ and T-type Ca$^{2+}$ currents were inactivated by a 200-ms conditioning prepulse to −40 mV, and $I_{\text{Ca}}$ through L-type Ca$^{2+}$-channels was elicited by a depolarizing test pulse to +10 mV; holding potential was −80 mV.) Pipette solutions contained 1 mM Mg$^{2+}$ (pMg = 3; A) and 1 μM Mg$^{2+}$ (pMg = 6; B).

**Fig. 2.** GTP blocks the enhancing effect of low-Mg$^{2+}$ internal solution (pMg of 5 and 6) via the whole cell patch pipette caused an increase in peak $I_{\text{Ca}}$ along a biphasic time course, i.e, an early increase in $I_{\text{Ca}}$ at 28°C in the absence of GTP ($I_{\text{Ca}}$) than in the presence of GTP (0.4 mM; E). Each curve gives the time course of $I_{\text{Ca}}$ in an individual cell. The GTP effect was reproducible in several trials. In two of the trials with low Mg$^{2+}$ (pMg of 6) plus GTP in the pipette solution, the temperature was changed in two steps, from 24 to 30°C and from 30 to 32°C.

*Statistical significance ($P < 0.01$) between the groups, pMg6 and pMg6 + GTP.

**Mg$^{2+}$ concentration dependence of $I_{\text{Ca}}$.** At 32°C, intracellular dialysis of low-Mg$^{2+}$ solution (pMg of 5 and 6) via the whole cell patch pipette caused an increase in peak $I_{\text{Ca}}$ along a biphasic time course, i.e, an early increase in $I_{\text{Ca}}$ at 28°C in the absence of GTP ($I_{\text{Ca}}$) than in the presence of GTP (0.4 mM; E). Each curve gives the time course of $I_{\text{Ca}}$ in an individual cell. The GTP effect was reproducible in several trials. In two of the trials with low Mg$^{2+}$ (pMg of 6) plus GTP in the pipette solution, the temperature was changed in two steps, from 24 to 30°C and from 30 to 32°C.

*Statistical significance ($P < 0.01$) between the groups, pMg6 and pMg6 + GTP.
transient increase (runup) and a later sustained increase (Fig. 3A). As runup was measured at the maximum I_{Ca} attained earlier than 6 min for each [Mg^{2+}], there was a slight [Mg^{2+}] dependency (Fig. 3C). The late phase of I_{Ca} increase was clearly [Mg^{2+}] dependent. Dialysis with solutions containing relatively high [Mg^{2+}] (pMg values of 2, 3, or 4) never induced the late phase of I_{Ca} increase. However, at pMg = 5, I_{Ca} was increased to a level comparable with that at pMg = 6 (Fig. 3A). The relationship between the Mg^{2+} concentration and the late phase of increased I_{Ca} is summarized in Fig. 3C. I_{Ca} in the late phase was measured at 12–14 min, the length of time required for I_{Ca} to reach a plateau at pMg = 5 and pMg = 6.) The current-voltage relationship (current-voltage curve) of I_{Ca} with pMg = 6 at Fig. 3A, a and b, was compared in Fig. 3B. Depletion of Mg^{2+} caused an increase in I_{Ca} as well as a negative shift of the current-voltage curve.

Phosphorylated Ca^{2+} channel does not respond to changes in [Mg^{2+}]. To induce maximal phosphorylation of L-type Ca^{2+} channels, we combined extracellular perfusion of 3 μM FSK with intracellular dialysis of the phosphatase inhibitor 10 μM okadaic acid (OA) throughout experiments. In agreement with previous findings in frog ventricular myocytes (29), this maneuver abolished the biphasic time course of I_{Ca} potentiation, which otherwise occurred during dialysis of low [Mg^{2+}] (pMg = 5 and 6) solutions (Fig. 3A). Thus it is conceivable that depletion of Mg^{2+} led to channel dephosphorylation when OA was not included in intracellular solutions, causing a decrease in I_{Ca}. With the use of this protocol, we monitored peak I_{Ca} through presumably fully phosphorylated Ca^{2+} channels during intracellular dialysis of various [Mg^{2+}] solutions and obtained curves of Mg^{2+} concentration dependence for the late phases of I_{Ca} potentiation from data at 11.2 min, as shown in Fig. 4A (open circles in Fig. 4B). The curve was not influenced by changes in [Mg^{2+}], until [Mg^{2+}] was raised to at least 10 mM. For comparison, the curve of Mg^{2+} concentration versus I_{Ca} (late phase) obtained in the absence of FSK and OA (closed squares in Fig. 4B) is replotted from Fig. 3C. This clearly indicates that phosphorylation abolished or markedly reduced the sensitivity of the Ca^{2+} channel to Mg^{2+} block, much as was previously demonstrated for the L-type Ca^{2+} channel in frog ventricular myocytes (29).

Early transient increase in I_{Ca} initiated by dialysis of patch pipette solutions. After the whole cell patch-clamp configuration was established, I_{Ca} gradually increased (runup) and reached a peak within 5 min at 24°C (Fig. 1B and Fig. 5A). Cell rupture, which permits intracellular dialysis with pipette solutions, was a requirement for runup, because we normally did not observe this phenomenon using the nystatin-perforated patch technique (4.98 ± 1.7 pA/pF at 1.2 min vs. 5.0 ± 1.5 pA/pF at 5.4 min for frog ventricular myo-

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![Graph](http://www.ajpheart.org)
Depletion of Mg$^{2+}$ may be a causative factor in runup, because Mg$^{2+}$ would be expected to diffuse into the pipette fluid down its electrochemical gradient, resulting in a reduction of Mg$^{2+}$ block. If this were the case, peak values of $I_{Ca}$ during runup should depend on the [Mg$^{2+}$] of the pipette solution. Thus we compared peak $I_{Ca}$ values as a function of the pipette [Mg$^{2+}$] at 24°C (Fig. 5B). There was a clear dependency of the early transient increase in $I_{Ca}$ on [Mg$^{2+}$] at 24°C.

In summary, we observed two forms of Mg$^{2+}$-dependent $I_{Ca}$ potentiation: an early transient increase (runup) at 24°C and a late phase of increase at 32°C. How can we distinguish between these two forms of potentiation? The mechanisms underlying the early and late phase of $I_{Ca}$ potentiation differ in their sensitivity to GTP. Hence, GTP (0.4 mM) suppressed the late phase of the $I_{Ca}$ increase but did not suppress the early phase, as shown in Fig. 5B (i.e., there was no significant difference between filled square and open circle values at pMg = 6). However, we needed to show that an effective dose of GTP was present at the time of early phases before concluding that the early phase of $I_{Ca}$ potentiation is GTP insensitive. We assayed the dialysis efficiency of cAMP from the pipette to the cell by observing increases in $I_{Ca}$, because the molecular...
size of cAMP is similar to GTP and diffusion of cAMP can be detected by an increase in $I_{Ca}$. As shown in Fig. 6A, an increase in $I_{Ca}$ saturated within 8 min with 0.4 mM cAMP, whereas it took 12 min with 0.1 mM cAMP. The time constants of the cAMP-dependent increase in $I_{Ca}$ were calculated to be 90.2 s for 0.4 mM cAMP and 396.6 s for 0.1 mM cAMP (in this calculation, the basal runup effect without cAMP, shown as control in Fig. 6, was canceled). As we compared the saturated values of $I_{Ca}$, $I_{Ca}$ with 0.4 mM cAMP reached the level of maximal $I_{Ca}$ with 0.1 mM cAMP within 2 min. This confirms that constituents of the 0.4 mM concentration in the pipette having a molecular weight similar to cAMP can reach to 0.1 mM in the cell within 2 min. In Fig. 6B, runup at 24°C with a pipette solution of pMg = 5 reached a peak around at 4 min. Similarly, runup with a pipette solution of pMg = 5 having 0.4 mM GTP reached peak at 4 min, and its peak amplitude completely coincided to that without GTP, where at least 0.1 mM GTP was supposed to be present in the cell. It clearly tells that this Mg$^{2+}$-sensitive early transient increase in $I_{Ca}$ is insensitive to GTP.

As to the runup, one may further argue that it might be Ca$^{2+}$ dependent, because cells perfused with Na$^+$-free external solution could be overloaded with Ca$^{2+}$ and dialysis with pipette solutions relieved Ca$^{2+}$ overload, thereby leading to reduction in Ca$^{2+}$-dependent inactivation. Such a possibility was tested by conducting experiments similar to those shown in Fig. 6B but under 100% Na$^+$ external solution, where Na$^+$ currents were suppressed by a combination of 3 μM TTX and depolarized conditioning pulses (see MATERIALS AND METHODS). Runup still existed in 100% Na$^+$ external solution, and low Mg$^{2+}$ (pMg = 5) cell dialysis facilitated runup (Fig. 6C). These results exclude the possible involvement of a Ca$^{2+}$ unload mechanism in causing runup when Na$^+$-free external solution was used.

Low Mg$^{2+}$ effect on frog $I_{Ca}$ at reduced temperature.

One of the unique properties of the guinea pig L-type Ca$^{2+}$ channel is that the response to [Mg$^{2+}$], depletion disappeared at low temperature. We determined whether L-type Ca$^{2+}$ channels in frog ventricular myocytes also possess such a property. In the amphibian myocyte, Mg$^{2+}$ sensitivity was present at room temperature. Therefore, we examined the effect of low Mg$^{2+}$ (pMg = 6) on $I_{Ca}$ at 17.5°C. In all fourteen trials at 17.5°C, low-Mg$^{2+}$ solution either failed to increase $I_{Ca}$ at all or induced a very small change. Averaged $I_{Ca}$ at 17.5°C obtained 12.1 min after the disruption of the membrane was $6.1 \pm 0.85$ pA/pF ($n = 14$), which is clearly smaller than that obtained at 24°C ($53.6 \pm 4.5$ pA/pF, $n = 10$). Estimated $I_{Ca}$ at 24°C from the value at

![Fig. 6](http://www.ajpheart.org/) Evaluation of distinct features of early transient increase in $I_{Ca}$ in guinea pig ventricular myocytes. A: efficiency of cAMP dialysis contained in patch pipettes into guinea pig ventricular myocytes. $I_{Ca}$ increased as cAMP diffused into cells in a dose-dependent manner. Data are presented as means ± SE of $I_{Ca}$. All data were taken at 24°C using pMg3 pipette solutions but without cAMP for control ($n = 4$), 0.1 mM cAMP ($n = 4$), and 0.4 mM cAMP ($n = 4$) added. B: intracellular GTP effect on runup facilitated by pMg5 solution. Data are presented as means ± SE of $I_{Ca}$. Data were obtained with pipette solutions of pMg5 with ($\bullet$; n = 4) and without 0.4 mM GTP ($\square$; n = 4). There was no statistical difference between these two groups. C: runup under 100% Na$^+$ external solution. Data were obtained with pMg3 ($n = 5$) and pMg5 ($n = 6$) solutions and are presented as means ± SE of $I_{Ca}$. In both conditions, runup was clearly observed and was larger with low Mg$^{2+}$. 

*AJP-Heart Circ Physiol* • VOL 282 • MARCH 2002 • www.ajpheart.org
17.5°C (6.1 pA/pF) using a Q\textsubscript{10} of pMg = 3 solution (2.05 ± 0.36, n = 4) for frog ventricular myocytes was 9.7 pA/pF, and this value was far below the one experimentally obtained with the low-Mg\textsuperscript{2+} (pMg = 6) solution (53.6 pA/pF). The protocol depicted in Fig. 1 was applied to frog myocytes with the exception that the temperature change was between 17.5 and 24°C; the results are shown in Fig. 7. Similar to findings in guinea pig ventricular myocytes, raising the temperature from 17.5 to 24°C produced a much larger effect at pMg = 6 (Q\textsubscript{10} = 23.0 ± 6.9, n = 6) than at pMg = 3 (Q\textsubscript{10} = 2.05 ± 0.36, n = 4) or at pMg = 6 (Q\textsubscript{10} = 3.3, n = 2) with GTP present. Moreover, the lack of reversibility of the low Mg\textsuperscript{2+} effect was the same as our finding in guinea pig myocytes.

Temperature coefficients for various parameters of I\texttextsubscript{Ca}. The Q\textsubscript{10} value for time to peak I\texttextsubscript{Ca} was −3.0 in all groups of guinea pig myocytes tested (see Fig. 8B). The effects of temperature change on inactivation kinetics were more complicated. At pMg = 6 or in the presence of FSK, I\texttextsubscript{Ca} decayed along a double-exponential time course (with time constant $\tau_1\text{inact}$ and $\tau_2\text{inact}$), but at pMg = 3, I\texttextsubscript{Ca} decayed monexponentially (with time constant $\tau\text{inact}$). The Q\textsubscript{10} value for $\tau\text{inact}$, which averaged 5.6 ± 0.2 ms (n = 5), was obtained from the data shown in Fig. 8C. For other conditions (pMg = 6 and FSK stimulation), faster ($\tau_1\text{inact}$) and slower ($\tau_2\text{inact}$) time constants and their relative amplitudes are shown in the three-dimensional plot shown in Fig. 9. The relative amplitude for $\tau_1\text{inact}$ was increased and that for $\tau_2\text{inact}$ was decreased by the rise in temperature in both experimental conditions, whereas the temperature effect on FSK-stimulated current was much smaller. The same temperature increase diminished both $\tau_1\text{inact}$ and $\tau_2\text{inact}$ at pMg = 6, but this kind of effect was absent or much smaller in the case of FSK stimulation. Thus despite the complexity of analyzing the temperature dependency of I\texttextsubscript{Ca}, decay (particularly in experimental conditions yielding double-exponential kinetics), the results indicate that a rise in temperature accelerates the inactivation process. The relatively small temper-
The Q10 value for peak \( I_{\text{Ca}} \), which averaged 14.5 ± 0.2 (\( n = 6 \)), was peculiar to the low \( \text{Mg}^{2+} \) condition (\( \text{pMg} = 6 \)). This value is quite different from the values obtained at \( \text{pMg} = 3 (3.7 ± 0.4, n = 4) \) or \( \text{pMg} = 6 \) with GTP present (3.9 ± 0.4, \( n = 4 \)) (Fig. 8A) and is at variance with previously reported results (2, 7).

**DISCUSSION**

We found that \( \text{Mg}^{2+} \)-dependent inhibition of the L-type \( \text{Ca}^{2+} \) channel is present in both guinea pig and frog cardiomyocytes and is temperature dependent. The only difference between the frog and the guinea pig preparation was the temperature range capable of activating this \( \text{Mg}^{2+} \)-dependent mechanism; i.e., the mechanism is activated above 32°C in guinea pig myocytes and above 24°C in frog myocytes. The remarkably high Q10 value (14.5) for low \( \text{Mg}^{2+} \) potentiation (\( \text{pMg} = 6 \)) of the \( I_{\text{Ca}} \) amplitude compared with the low Q10 value for L-type \( \text{Ca}^{2+} \) channel kinetics may be evidence for a series of temperature-sensitive, enzymatic steps mediating the low \( \text{Mg}^{2+} \) effect.

Two kinds of \( \text{Mg}^{2+} \)-dependent regulation. At low \([\text{Mg}^{2+}]_i\), we observed two kinds of \( I_{\text{Ca}} \) facilitation: a transient early phase (runup) (taking place within <5 min from the start of intracellular dialysis) and a late phase (developing after >12 min). Both phases were \( \text{Mg}^{2+} \) sensitive but distinct from each other in that the \( \text{Mg}^{2+} \) dependency was much more manifested at low temperatures for runup and only at higher temperatures for the late phase of the \( I_{\text{Ca}} \) increase. Furthermore, the former mechanism was refractory to the blocking effect of GTP.

What is the mechanism for the marked temperature effect on peak \( I_{\text{Ca}} \) with low-\( \text{Mg}^{2+} \) solutions? Increasing temperature generally facilitates all kinetic processes. However, the final outcome of a temperature change in a complex system depends on the temperature sensitivity of each element in the system. For example, given similar Q10 values for the activation and inactivation kinetics of voltage-gated \( \text{Na}^{+} \) channels, we would predict, using the Hodgkin-Huxley model (10), only a small increase in peak current, i.e., faster channel inactivation tends to offset faster channel activation, and a gain in peak current would be expected solely on the basis of the slight temperature dependency of the single channel conductance, which is essentially the same as that of diffusion (Q10 value, ~1.5). In reality, it has been reported that the change in the open probability of L-type \( \text{Ca}^{2+} \) channels contributes to the decrease in \( I_{\text{Ca}} \) by the fall in temperature at a Q10 of 1.5 (13, 16) in addition to a factor of unit conductance. However, the Q10 value for peak \( I_{\text{Ca}} \) (14.5) with low-\( \text{Mg}^{2+} \) (\( \text{pMg} \) of 6) solutions, as found in the present study, is still extraordinary. This Q10 value cannot be explained by a single factor such as diffusion, which lacks the necessary temperature sensitivity. Thus several factors such as enzymatic processes, lipid membrane properties, changes in channel protein conformation, or coupling between L-type \( \text{Ca}^{2+} \) channels and other proteins (e.g., sarcoplasmic reticulum \( \text{Ca}^{2+} \) channels) could be involved in this phenomenon. Allen and Mikala (3) reported on the enhanced sensitivity of human L-type \( \text{Ca}^{2+} \) channels to temperature when they were expressed in \textit{Xenopus} oocytes, indicating the involvement of many factors such as membrane environment, channel assembly, and so on.

Such large values for the Q10 of voltage-dependent \( \text{Ca}^{2+} \) channels were also observed, albeit at relatively low temperatures (between 12.5 and 18.5°C), in mouse neuroblastoma cells (17). The temperature-sensitive mechanism in this case may be specific to neuroblas-
Fig. 9. Temperature effect on fast ($\tau_{1\text{inact}}$) and slow $\tau$ of inactivation ($\tau_{2\text{inact}}$) in guinea pig ventricular myocytes. $I_{\text{Ca}}$ decay was fitted with a double-exponential function at pMg = 6 (A) or at pMg = 3 with external FSK (B). The two $\tau$ and their relative amplitudes were obtained at temperatures of 24 or 32°C and plotted in three-dimensional format. A: at pMg = 6, temperature rise decreased $\tau_{1\text{inact}}$ and $\tau_{2\text{inact}}$ from 46.5 ± 29.8 and 129 ± 27 ms to 10.1 ± 0.98 and 33.3 ± 5.1 ms, respectively ($n = 3$). B: with phosphorylation stimulated by FSK, the initial values of $\tau_{1\text{inact}}$ and $\tau_{2\text{inact}}$ were relatively small, and the effect of temperature rise (from 24 to 32°C) was also minimal, i.e., $\tau_{1\text{inact}}$ and $\tau_{2\text{inact}}$ were changed from 7.8 ± 4.2 and 67.8 ± 5.8 ms to 9.5 ± 1.8 and 37.0 ± 2.2 ms, respectively ($n = 3$).

Rigidity of membranes with addition of Mg$^{2+}$ temperature at which phase transition occurs fitoma cells, because the pipette solution that Narahashi et al. (17) used contained high Mg$^{2+}$ (2.5 mM).

Another notable feature of our low Mg$^{2+}$ effect was that, compared with the Q10 value for $I_{\text{Ca}}$ potentiation (Q10 = 14.5), the Q10 for the reverse reaction was much lower (Q10 = 2.36 ± 0.31; see Figs. 1B and 7B). This kind of irreversibility cannot be explained in simple thermodynamic terms. It is possible that a membrane structure favoring an inactive channel state could be lost under low-Mg$^{2+}$ conditions in a critical temperature range.

Lipid membrane fluidity abruptly changes at certain transition temperatures and may be one of the mechanisms affecting the temperature dependency of channel function in conditions such as low Mg$^{2+}$. The specific temperature at which phase transition occurs depends on the composition of the lipid membrane (i.e., ratio of unsaturated to saturated phospholipid acyl chains and the lipid class composition) (5). It has been suggested that the electrical function of nerve cell membranes in poikilotherms shows temperature acclimation (or homeoviscous adaptation) by maintaining relatively constant membrane fluidity (14). If membrane fluidity modulates the function of membrane proteins, it is reasonable to expect a different optimum temperature for the low Mg$^{2+}$ effect in guinea pig and frog ventricular myocytes, as we observed in this study. A body of examples for divalent cation effects on membrane fluidity can be seen in a variety of lipid membranes (4, 15, 24). Mitochondrial H$^+$-ATPase activities contained in liposomes are activated in the presence of Mg$^{2+}$ due to Mg$^{2+}$-induced alteration of membrane fluidity (11, 30, 31). In the erythrocyte membrane, rigidified membranes with addition of Mg$^{2+}$ were detected by fluorescence polarization studies (24). However, even with these examples, we are still unable to account for the relief from Mg$^{2+}$ block only at high temperatures seen in this study. Further investigation into channel function during the experimental manipulation of membrane fluidity will be needed to provide evidence for such a possibility.

Inactivation kinetics vary with the internal environment. Generally speaking, current decay of the L-type Ca$^{2+}$ channel in guinea pig ventricular myocytes proceeds along a double-exponential time course (2, 6, 23, 33). In this study, we found that fixing [Mg$^{2+}$]$_i$ at 1.0 mM (using a strong Ca$^{2+}$ buffer of BAPTA and Mg$^{2+}$ buffers, EDTA, and ATP) eliminated the faster time constant, leading to a monoexponential decay. This special state was perturbed by phosphorylation or reduction in [Mg$^{2+}$], i.e., either treatment created a faster component of inactivation. Do these two mechanisms (phosphorylation and Mg$^{2+}$ depletion) ultimately cause the same effects on inactivation? There is a discrepancy of values of time constants between an pMg of 6 and 3 (FSK) at 24°C (Fig. 9). However, $\tau_{\text{inact}}$ at pMg = 6 are quite close to those at pMg = 3 (FSK) at 32°C. This is rather likely, because $I_{\text{Ca}}$ of guinea pig ventricular myocytes was not stimulated with Mg$^{2+}$-depleting solution at 24°C as shown so far. Thus we can reasonably argue that enhanced $I_{\text{Ca}}$ stimulated either with Mg$^{2+}$ depletion or FSK show a similar $\tau_{\text{inact}}$. These results are consistent with our hypothesis that phosphorylation modulates the channel activity by changing sensitivity to Mg$^{2+}$ block, because phosphorylation produced a kinetic effect similar to that of low Mg$^{2+}$.

Phosphorylation and temperature. The temperature effect on phosphorylated Ca$^{2+}$ channels was twofold. First, channel phosphorylation is regulated by the balance between two distinct enzyme types, protein kinases and protein phosphatases. The temperature dependences of these enzymatic activities are different, so that maximal channel phosphorylation would be achieved at a temperature optimal for net phosphorylation. Second, the activating energy required to open the channel may be lower for phosphorylated channels. Thus the thermodynamic effect would be greater for unphosphorylated channels. This was the case in our study, because the Q10 values for peak $I_{\text{Ca}}$ were smaller in phosphorylated channels, which is consistent with Allen’s results (2).

Physiological relevance. As seen in this study, temperature was an important factor in the regulation of the L-type Ca$^{2+}$ channel in both mammalian and amphibian cardiac myocytes. Thus one must be careful to avoid ruling out mechanisms in a system without considering the influence of temperature. At the same time, the regulatory machinery of the L-type Ca$^{2+}$ channel is not simple but is a well organized and
orderly system influenced by many factors, including lipid structure, combinations of different kinds of enzymes, and so on.

Finally, this report has considered the physiological role of Mg\(^{2+}\)-dependent block in the functioning of L-type Ca\(^{2+}\) channels in mammalian cardiac myocytes. We previously postulated that Mg\(^{2+}\)-dependent block in frog ventricular myocytes may be a key regulatory step in recruitment of L-type Ca\(^{2+}\) channels through phosphorylation, because this Mg\(^{2+}\)-dependent block was occluded when cell membranes were phosphorylated (29). We observed, in the present study, the same phenomenon in guinea pig ventricular myocytes at temperatures above 32 °C.

We now want to identify the mechanism directly responsible for the temperature effect, which should facilitate our further understanding of the phosphorylation event regulating the L-type Ca\(^{2+}\) channel.

We thank Dr. Stephen M. Vogel (Department of Pharmacology, University of Illinois at Chicago, College of Medicine) for critical reading of the manuscript. This work was supported by Ministry of Education and Culture of Japan Grant 11470011 (to K. Yamaoka).

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