Effects of Intracellular Free Magnesium on Calcium Current in Isolated Cardiac Myocytes

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Magnesium ions play a fundamental role in cellular function, but the effects of changes in the concentration of intracellular ionized magnesium ($[Mg^{2+}]_i$) on cell physiology have only recently received experimental attention. Increasing $[Mg^{2+}]_i$ from 0.3 to 3.0 m*M* in cardiac cells by internal perfusion has only small effects on the basal voltage-gated calcium current (I_{Ca}) or on I_{Ca} elevated by dihydropyridine calcium channel agonists. In contrast, I_{Ca} elevated by cyclic adenosine monophosphate (cAMP)–dependent phosphorylation decreases by more than 50 percent. The effect of $[Mg^{2+}]_i$ is not due to changes in the concentration of cAMP or in the velocity of phosphorylation but rather appears to be a direct effect on the phosphorylated channel or on channel dephosphorylation.

THE VOLTAGE-GATED CALCIUM CURRENT, I_{Ca}, plays a key role in the development of the cardiac action potential (*1*), in the generation of electrical pacemaking in nodal tissue (*2*), and in the initiation of contraction (*3*). I_{Ca} is modulated by cyclic adenosine monophosphate (cAMP)–dependent protein phosphorylation by neurotransmitters and drugs that influence β-adrenergic and cholinergic receptors on cardiac cells (*1, 4, 5*). Other intracellular constituents that might affect or regulate I_{Ca} in cardiac cells, however, have not received much attention. We have examined the effects of changes in the concentration of intracellular free magnesium ($[Mg^{2+}]_i$) on I_{Ca} because magnesium ions are an essential cofactor in hundreds of enzymatic reactions, have a large effect on certain potassium channels (*6*), and probably play a role in cellular regulation (*7, 8*). We show that changes in $[Mg^{2+}]_i$ have a major effect on the amplitude of I_{Ca} elevated by cAMP-dependent mechanisms but that this effect is not mediated by changes in intracellular cAMP concentration.

We measured I_{Ca} in single isolated myocytes from frog ventricle by using the whole-cell configuration of the patch clamp technique (*9*) as described (*4*). Sodium current was blocked with tetrodotoxin, and potassium currents were blocked with both internal and external cesium. I_{Ca} was measured as the net inward current (peak current minus steady-state current at the end of the 400-msec voltage pulse) in response to a voltage pulse to 0 mV from a holding potential of −80 mV.

The $[Mg^{2+}]_i$ was changed during recording by a system that permitted continuous perfusion of the patch electrode as previously described (*4*). The composition of the internal solutions was calculated by a computer program provided by Godt and Lindley (*10*). The use of this program allowed us to maintain a constant MgATP concentration while varying $[Mg^{2+}]$. Concentrations of Mg^{2+} between 0.3 m*M* and 3.0 m*M* were used because the best estimates for $[Mg^{2+}]_i$ in muscle lie in this range (*8, 11*).

We examined the effect of $[Mg^{2+}]_i$ on cardiac β-adrenoceptor–elevated I_{Ca} by superfusing cells with isoproterenol (1.0 μ*M*) in the presence of low (0.3 m*M*) or high (3.0 m*M*) $[Mg^{2+}]_i$. Changing $[Mg^{2+}]_i$ from 0.3 m*M* to 3.0 m*M* caused a significant reduction in the isoproterenol-stimulated I_{Ca} (Fig. 1A). When $[Mg^{2+}]_i$ was returned to 0.3 m*M*, I_{Ca} increased. Analysis of the data was somewhat complicated by the run-down of I_{Ca} that occurred. Nevertheless, in three cells in which a reversible effect of changing $[Mg^{2+}]_i$ was achieved, the amplitude of the isoproterenol-elevated I_{Ca} in 0.3 m*M* $[Mg^{2+}]_i$ was 88 ± 5.7% larger than I_{Ca} in 3.0 m*M* $[Mg^{2+}]_i$ (Table 1). This effect of $[Mg^{2+}]_i$ on the isoproterenol-elevated I_{Ca} could be due to an effect on several Mg^{2+}-requiring steps between β-receptor activa-

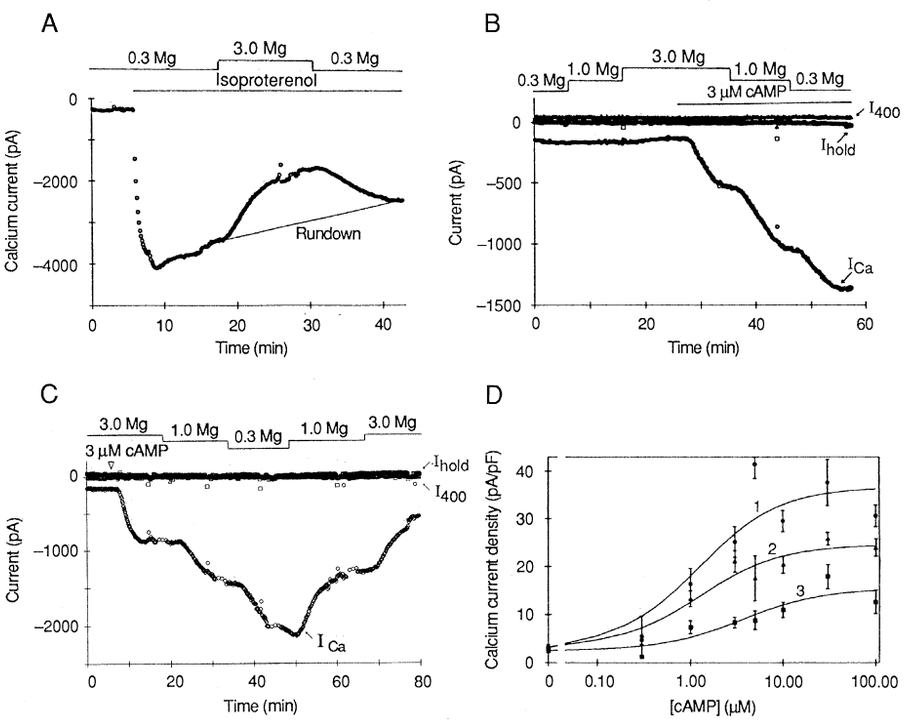

Fig. 1. Effect of changing $[Mg^{2+}]_i$ on I_{Ca}. Cells were bathed in Cs-Ringer solution containing 88 m*M* NaCl, 20 m*M* CsCl, 0.6 m*M* Na_2PO_4, 24 m*M* $NaHCO_3$, 1.8 m*M* $MgCl_2$, 1.8 $CaCl_2$, 5 m*M* sodium pyruvate, 5 m*M* glucose, 0.3 μ*M* tetrodotoxin, *p*H 7.4, with 95% O_2 and 5% CO_2, and perfused internally with the solutions in (*10*). In (A) through (C) circles represent net I_{Ca} elicited by a 400-msec pulse to 0 mV from a holding potential of −80 mV. Triangles represent the current at the end of the 400-msec pulse (I_{400}). Squares represent the holding current at −80 mV. (**A**) Effect of $[Mg^{2+}]_i$ on the response to isoproterenol. Starting at t = 7 minutes, the cell was exposed extracellularly to 1 μ*M* isoproterenol and perfused internally with solutions containing calculated $[Mg^{2+}]$ of 0.3 m*M* or 3.0 m*M* as indicated. Increasing $[Mg^{2+}]_i$ to 3.0 m*M* caused a decrease in I_{Ca} that was reversible upon returning to 0.3 m*M* $[Mg^{2+}]_i$, on the assumption that I_{Ca} would have continued to run down at a constant rate. (**B**) Effect of $[Mg^{2+}]_i$ on basal I_{Ca} and on I_{Ca} elevated by 3 μ*M* cAMP. The cell was bathed in Cs-Ringer solution for the entire experiment and perfused internally with solutions containing 0.3 m*M*, 1.0 m*M*, or 3.0 m*M* $[Mg^{2+}]$ as shown. (**C**) Reversibility of $[Mg^{2+}]_i$ effect on I_{Ca} elevated by 3 μ*M* cAMP. The cell was initially perfused internally with solution containing 3.0 m*M* $[Mg^{2+}]_i$. At t = 8 minutes, internal perfusion of the cell with 3 μ*M* cAMP was begun, and $[Mg^{2+}]_i$ was changed as indicated. (**D**) Concentration-response relation for the effect of internally perfused cAMP on I_{Ca} in the presence of different $[Mg^{2+}]_i$. Curve 1 represents 0.3 m*M* $[Mg^{2+}]_i$; curve 2, 1.0 m*M* $[Mg^{2+}]_i$; and curve 3, 3.0 m*M* $[Mg^{2+}]_i$. I_{Ca} density was calculated by dividing the net calcium current by the cell capacitance. Each point is the mean of 3 to 48 cells; bars represent SEM.

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tion and channel phosphorylation. To characterize this phenomenon further, we examined the effect of $[Mg^{2+}]_i$ on basal I_{Ca} and on I_{Ca} elevated by intracellular perfusion with cAMP.

Changing the internal perfusion solution from one containing 0.3 m*M* $[Mg^{2+}]$ to one containing 1.0 m*M* $[Mg^{2+}]$ had little effect on basal I_{Ca} (Fig. 1B). Increasing $[Mg^{2+}]$ further (3.0 m*M*) caused I_{Ca} to decrease slightly. On average, basal I_{Ca} with 0.3 m*M* $[Mg^{2+}]_i$ was 26% greater than I_{Ca} with 3.0 m*M* $[Mg^{2+}]_i$ (Table 1).

In contrast to basal I_{Ca}, I_{Ca} elevated by intracellular perfusion with cAMP more than doubled as $[Mg^{2+}]_i$ was decreased from 3.0 m*M* to 0.3 m*M* (Fig. 1B). On average, decreasing $[Mg^{2+}]_i$ from 3.0 m*M* to 0.3 m*M* increased by 180% the I_{Ca} that had been elevated by 10 μ*M* cAMP (Table 1). $[Mg^{2+}]_i$ had no effect on holding current or on the current at the end of the pulse (I_{400}). The effect of changing $[Mg^{2+}]_i$ occurred rapidly; the time course of change was similar to the time course of the increase in I_{Ca} produced by intracellular perfusion with cAMP. The effect of changing $[Mg^{2+}]_i$ was usually reversible (Fig. 1C).

The effect of $[Mg^{2+}]_i$ on cAMP-elevated I_{Ca} is further illustrated by the concentration-response relation for cAMP (Fig. 1D). With lower $[Mg^{2+}]_i$, the amplitude of I_{Ca} was greater at all cAMP concentrations. The data for each $[Mg^{2+}]_i$ in Fig. 1D were fitted to an equation of the form $I_{Ca} = I_{max} \times [cAMP]/(EC_{50} + [cAMP])$, to obtain the maximum I_{Ca} (I_{max}) and the mean effective concentration (EC_{50}) for cAMP. The calculated I_{max} values in 0.3 m*M*, 1.0 m*M*, and 3.0 m*M* $[Mg^{2+}]_i$ were 36.6, 24.6, and 15.4 pA/pF, respectively. The effect of $[Mg^{2+}]_i$ on EC_{50}, however, was questionable; the EC_{50} values were 1.24, 1.28, and 3.65 μ*M* for 0.3, 1.0, and 3.0 m*M* $[Mg^{+2}]_i$, respectively.

One possible mechanism that could explain these results is that $[Mg^{2+}]$ alters the activity of phosphodiesterases (PDEs) that hydrolyze cAMP (*12*). We tested this hypothesis by examining the effect of $[Mg^{2+}]_i$ on I_{Ca} elevated by intracellular perfusion with 8-bromo-cAMP (8Br-cAMP), a nonhydrolyzable analog of cAMP. I_{Ca} elevated by 10 μ*M* 8Br-cAMP was decreased reversibly about 60% by increasing $[Mg^{2+}]_i$ from 0.3 m*M* to 3.0 mM (Fig. 2A). In three cells, I_{Ca} density in the presence of 10 μ*M* 8Br-cAMP was 124% greater with 0.3 m*M* $[Mg^{2+}]_i$ than with 3.0 m*M* $[Mg^{2+}]_i$ (Table 1). Thus, the inhibitory influence of intracellular Mg^{2+} on cAMP-elevated I_{Ca} could not be attributed solely to enhancement of PDE activity.

The above results suggested that the effect of $[Mg^{2+}]_i$ was exerted primarily on a site other than those regulating intracellular cAMP. To test this hypothesis further, we determined the effect of $[Mg^{2+}]_i$ on I_{Ca} elevated by intracellular perfusion with catalytic subunit of cAMP-dependent protein kinase (A-PK) (Fig. 2B). Lowering $[Mg^{2+}]_i$ from 3.0 m*M* to 0.3 m*M* increased I_{Ca} by 140% in this particular cell. On average, I_{Ca} elevated by A-PK in 0.3 m*M* $[Mg^{2+}]_i$ was 125% larger than that recorded with 3.0 m*M* $[Mg^{2+}]_i$ (Table 1). Therefore, the inhibitory effect of Mg^{2+} was not primarily due to an action on cAMP concentration. Since A-PK activity is not significantly inhibited by $[Mg^{2+}]$ in this concentration range (*13*), the effect of $[Mg^{2+}]$ was not likely due to changes in velocity of channel phosphorylation unless $[Mg^{2+}]_i$ alters the ability of the calcium channel to act as a substrate for A-PK.

The final possibility we tested was whether $[Mg^{2+}]_i$ might affect the response to any agent that elevated I_{Ca}. The effect of altered $[Mg^{2+}]_i$ on I_{Ca} elevated by (+)202-791 (Sandoz), a dihydropyridine calcium chan-

Table 1. Effects of internal magnesium on calcium current amplitude. Values are means ± SEM, and the number of cells is shown in parentheses.

Cell treatment	I_{Ca} density (pA/pF) for $[Mg^{2+}]_i$ at		
	0.3 m*M*	1.0 m*M*	3.0 m*M*
Basal	3.18 ± 0.25 (48)	3.22 ± 0.54 (12)	2.52 ± 0.21 (32)
Isoproterenol (1.0 μ*M*)	42.73 ± 8.63 (3)		23.75 ± 4.55 (3)
cAMP (10 μ*M*)	29.5 ± 2.23 (8)	20.38 ± 1.80 (8)	10.96 ± 1.58 (7)
8Br-cAMP (10 μ*M*)	50.2 ± 4.7 (3)		22.4 ± 1.0 (3)
Protein kinase (A-PK)	34.2 ± 5.0 (3)		15.2 ± 0.5 (3)
(+)202-791	23.07 ± 1.95 (9)		17.16 ± 1.24 (9)

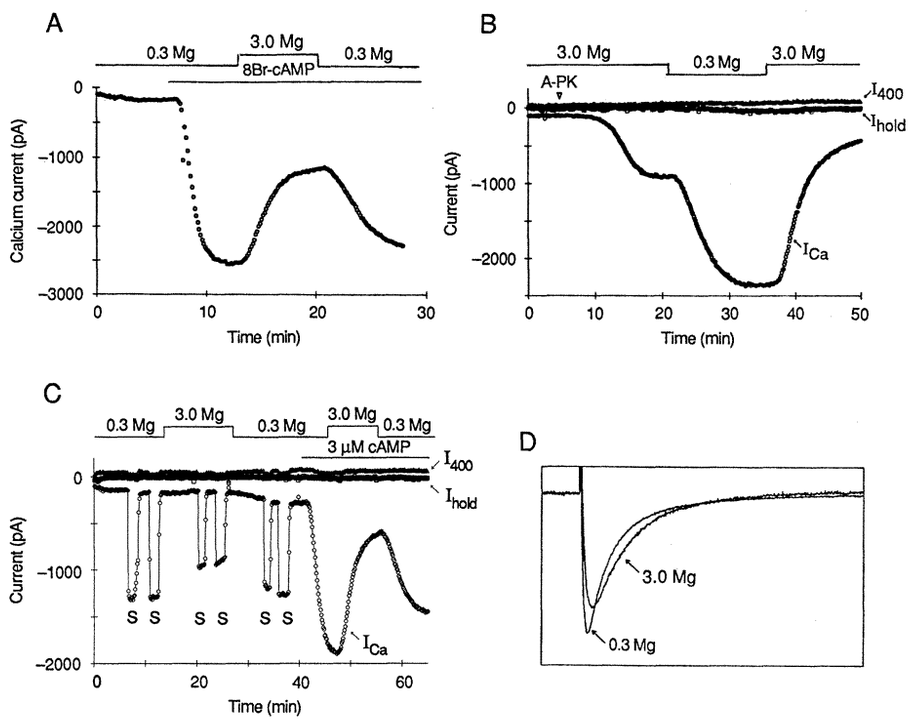

Fig. 2. Mechanism of effect of $[Mg^{2+}]_i$ on I_{Ca}. Symbols are the same as in Fig. 1B. (**A**) Effect of changing $[Mg^{2+}]_i$ on I_{Ca} elevated by internal perfusion with 8Br-cAMP. At t = 8 minutes, 10 μ*M* 8Br-cAMP was added to the internal perfusion. Increasing $[Mg^{2+}]_i$ to 3.0 m*M* for the period indicated caused a reversible decrease in I_{Ca}. (**B**) Effect of changing $[Mg^{2+}]_i$ on I_{Ca} elevated by internal perfusion with purified catalytic subunit of cAMP-dependent protein kinase (A-PK) (150 μg/ml) (*18*). Decreasing $[Mg^{2+}]_i$ to 0.3 m*M* caused a reversible increase in I_{Ca} elevated by the protein kinase. (**C**) Effect of changing $[Mg^{2+}]_i$ on I_{Ca} elevated by (+)202-791 (Sandoz). Initially the cell was perfused internally with 0.3 m*M* $[Mg^{2+}]_i$. Then (+)202-791 (2 μg/ml) was applied extracellularly during the periods marked "S." Increasing $[Mg^{2+}]_i$ from 0.3 m*M* to 3.0 m*M* caused a small decrease in I_{Ca} elevated by (+)202-791. At t = 40 minutes, 3 μ*M* cAMP was added to the internal perfusion, and $[Mg^{2+}]_i$ was changed as indicated. (**D**) Traces of I_{Ca} recorded with 3 μ*M* cAMP and either 0.3 m*M* $[Mg^{2+}]_i$ or 3.0 m*M* $[Mg^{2+}]_i$. Currents from Fig. 1C at t = 20 minutes and t = 45 minutes were digitized at 5 kHz and plotted. I_{Ca} in 0.3 m*M* $[Mg^{2+}]_i$ was 2100 pA, and I_{Ca} in 3.0 m*M* $[Mg^{2+}]_i$ was 875 pA. Current traces are 170 msec in duration.

nel agonist that elevates I_{Ca} by a cAMP-independent mechanism (*14*), is illustrated in Fig. 2C. This compound was applied extracellularly during the periods marked "S" in Fig. 2C, and $[Mg^{2+}]_i$ was varied as indicated. Although the effect of (+)202-791 on I_{Ca} decreased somewhat when $[Mg^{2+}]_i$ was increased from 0.3 m*M* to 3.0 m*M*, the decrease was not nearly as large as the decrease that occurred when I_{Ca} was elevated by 3 μ*M* cAMP in the same cell. In nine cells I_{Ca} elevated by (+)202-791 in the presence of 0.3 m*M* $[Mg^{2+}]_i$ was 32 ± 3.7% larger than I_{Ca} in the presence of 3.0 m*M* $[Mg^{2+}]_i$ (Table 1).

The fact that $[Mg^{2+}]_i$ has a small effect on basal I_{Ca} and on I_{Ca} elevated by (+)202-791 suggests that Mg^{2+} may bind to the channel to alter channel gating, but that the Mg^{2+} effect is more pronounced when the channel is phosphorylated. Evidence in support of this suggestion is that the kinetics (primarily the activation kinetics) of I_{Ca} are different in different $[Mg^{2+}]_i$ (Fig. 2D). Alternatively, $[Mg^{2+}]_i$ may regulate protein phosphatase activity. Some phosphatases, particularly protein phosphatase 2C, are known to be regulated by $[Mg^{2+}]$ in this range (*15*).

It is likely that $[Mg^{2+}]_i$ is regulated physiologically because $[Mg^{2+}]_i$ is far from thermodynamic equilibrium in cells, and its concentration changes under a variety of physiological conditions (*8, 11*). For this reason, we propose that $[Mg^{2+}]_i$ may play a role in the physiological regulation of I_{Ca}. Furthermore, the inhibitory effect of $[Mg^{2+}]_i$ on I_{Ca} could explain some of the pathological effects of magnesium deficiency in the heart (*16*). When processes that maintain low $[Ca^{2+}]_i$ are decreased (for example, during ischemia), increases in I_{Ca} produced by loss of $[Mg^{2+}]_i$ could contribute to calcium overload and myocardial damage (*17*).

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A Fossil Reptile Embryo from the Middle Triassic of the Alps

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The first nothosaur (*Neusticosaurus* sp.) embryo, one of the very few fossil embryos known, provides a rare glimpse at reproduction in extinct reptiles. The specimen from the southern Alpine Middle Triassic (about 230 million years ago) was recognized as an embryo in comparison with an exceptionally large and well-understood sample of juvenile and sexed adult *Neusticosaurus* sp. The skeleton shows many embryonic features and may well be the smallest fossil reptile known (body length 51 millimeters). It reached only 22% of mean adult length whereas modern reptiles of this size do not hatch before they reach about 30% of mean adult length. The question of ovipary versus vivipary in pachypleurosaurs is discussed in light of the embryo.

THE ONLY FOSSIL REPTILE EMBRYOS described until now were those of ichthyosaurs (*1*); dinosaur embryos in eggs have recently been found (*2*) but are not yet described. I report the first well-preserved nonichthyosaurian reptile embryo (Fig. 1) of the small pachypleurosaurid nothosaur *Neusticosaurus* sp. (*3*) from the famous Anisian and Ladinian (Middle Triassic, about 230 million years old) Monte San Giorgio bituminous shales (*4, 5*) of southern Switzerland and northern Italy. The specimen possibly is the smallest skeleton of an extinct reptile ever discovered and underscores the exceptional nature of this locality. Its importance is appreciated best in view of how little is known about reproduction in fossil reptiles. Eggs are only known from a few groups such as dinosaurs and turtles (*6, 7*), and embryos only from ichthyosaurs and now pachypleurosaurs.

Neusticosaurus is the most abundant fossil reptile from this locality; about 800 specimens are at the Paleontological Institute and Museum of the University of Zurich (PIMUZ). The mostly complete skeletons belong to two species that are clearly separated stratigraphically. *Neusticosaurus* sp. was a small (adult overall body length 230 to 370 mm), lizard-like inhabitant of warm, shallow coastal waters (*5*). In pachypleurosaurids, aquatic adaptation had not progressed as far as in plesiosaurs and ichthyosaurs, and the animals clearly were still able to leave the water (*3*).

Of the 97 prepared skeletons of *Neusticosaurus* sp., 24 are juveniles and furnish a complete growth series (Figs. 2 and 3). This large sample made sexing (sex A and B) and recognition of sexual maturity possible. The very homogeneous sample comes from a narrow stratigraphic interval and is treated as representing a single population. The

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