Magnesium Inhibits Norepinephrine Release by Blocking N-Type Calcium Channels at Peripheral Sympathetic Nerve Endings

Tatsuo Shimosawa, Koji Takano, Katsuyuki Ando, Toshiro Fujita

Abstract—Although Mg$^{2+}$ contributes to blood pressure regulation partly in terms of vasodilator action, its sympatholytic effect may also play an important role to control blood pressure. Thus, in the present study, we investigated the effect of Mg$^{2+}$ on sympathetic tone and blood pressure. We studied its actions on the blood pressure response to hydralazine, a direct vasodilator, in conscious spontaneously hypertensive rats (SHRs), and to electrical stimulation in the pithed Sprague-Dawley rat; catecholamine release by peripheral sympathetic nerve endings; and the N-type Ca$^{2+}$ channels of cultured neural cells. Intravenous Mg$^{2+}$ infusion (MgSO$_4$: 3×10$^{-6}$ mol/kg body weight/min) induced the greater hypotensive response to hydralazine with attenuated reflex tachycardia in SHRs. In pithed rats, Mg$^{2+}$ infusion significantly attenuated the blood pressure elevation (2±2 mm Hg versus 27±6 mm Hg, $P<0.01$) in response to spinal electrical stimulation. In the perfused mesenteric arteries system, norepinephrine release was significantly attenuated by high Mg$^{2+}$ concentration solution (4.8 mmol/L) compared with normal Mg$^{2+}$ solution (1.2 mmol/L). When we applied the perforated whole-cell patch clamp method to nerve growth factor-treated PC12 cells, Mg$^{2+}$ blocked voltage-gated Ca$^{2+}$ currents in a concentration-dependent manner. The majority of the voltage-gated Ca$^{2+}$ currents were carried through N-type channels, followed by L-type channels. Mg$^{2+}$ blocked both of these channels. These findings suggest that Mg$^{2+}$ blocks mainly N-type Ca$^{2+}$ channels at nerve endings, and thus inhibits norepinephrine release, which decreases blood pressure independent of its direct vasodilating action. *(Hypertension. 2004;44:897-902.)*

Key Words: hypertension ▪ catecholamines ▪ ion channels ▪ cocaine

The second most plentiful cation in intracellular fluid and an essential element for the activity of many enzymes is Mg$^{2+}$. In addition to these biochemical actions, magnesium salts have been shown to lower blood pressure. The hypotensive effect of Mg$^{2+}$ has been found to be mediated by nonspecific antagonism of the Ca$^{2+}$ channels in vascular smooth muscle cells. On the contrary, Mg$^{2+}$ deficiency results in a high catecholamine level, altered barofunction, and hypertension. Sympathoactivation and catecholamine elevation induce tachycardia, glucose intolerance, and abnormal fat metabolism, as well as direct cardiotoxicity. At the same time, recent clinical studies revealed that Mg$^{2+}$ protects brain from ischemic damages. The mechanism for neuroprotection of Mg$^{2+}$ is not fully elucidated; however, similar to Mg$^{2+}$ effect on vasculature, it is speculated that Mg$^{2+}$ induced decrease in intracellular Ca$^{2+}$ plays an important role. It can be hypothesized that Mg$^{2+}$ effect on Ca$^{2+}$ homeostasis in neural cells may be a common mechanism between sympathoinhibitory and neuroprotective action of Mg$^{2+}$.

In the present study, we performed in vivo and ex vivo experiments to investigate the effect of Mg$^{2+}$ on circulatory control by modulating sympathetic tone. Also, we investigated the in vitro effect of extracellular Mg$^{2+}$ concentrations on the voltage-gated Ca$^{2+}$ current of nerve growth factor (NGF)-differentiated PC12 cells, which have characteristics in common to peripheral sympathetic nerves.

Methods
For a detailed Methods section, please see http://hyper.hypertensionaha.org.
All protocols in this study were approved by the ethics committee of our institution, and rats were handled in our accredited facility in accordance with the institutional animal care policies of the University of Tokyo. All research protocols conformed to the guiding principles for animal experimentation as enunciated by the Ethics Committee on Animal Research of the University of Tokyo, Faculty of Medicine.

Protocol 1: Sympatholytic Effect of Mg$^{2+}$ and Blood Pressure Changes In Vivo
Male spontaneously hypertensive rats were used in this experiment. The rats were divided into 2 groups: Mg$^{2+}$ group (n=8) and a control group (n=7). In the Mg$^{2+}$ group, MgSO$_4$ (3×10$^{-6}$ mol/kg body weight/min) was infused, and in the control group saline was infused. Responses to hydralazine (1, 3, and 6×10$^{-5}$ g/kg body weight) were observed. A 100 μL blood sample was drawn before and after saline or MgSO$_4$ infusion to measure the serum Mg level. The nadir mean

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897
arterial pressure (MAP) and peak heart rate (HR) in 2 groups were compared. Male Sprague–Dawley rats were pithed as described elsewhere. Briefly, rats were pithed by inserting a steel rod covered with enamel except at its tip (5 mm), and its end was positioned at the level of the 7th to 10th thoracic vertebrae. The rats were divided into 2 groups as described. After infusion for 20 minutes, we stimulated at 0.5 Hz for 1 minute with an electrical stimulator between the pithing rod and the indifferent electrode. Each stimulus was 50 V and 1 millisecond. Blood pressure and HR changes were observed. To confirm quality of pithing, the rats were injected with 1 mg/kg hexamethonium, and responses to electrical stimulation were observed after the aforementioned experiments.

Protocol 2: Effect of Mg2+ on Norepinephrine Release by Peripheral Sympathetic Nerve Ending

The superior mesenteric artery from Sprague–Dawley rats was prepared by a modification of Castellucci method. The preparations were perfused with a Krebs–Henselit solution with 1.2 mmol/L of Mg2+. Low-Mg2+ buffer was prepared by reducing the MgSO4 concentration to 0.3 mmol/L substitution with Na2SO4, and high-Mg2+ buffer with a magnesium concentration of 3.6 mmol/L was prepared by replacing NaCl with MgCl, iso-osmotically.

A platinum electrode was placed around the periarterial plexus of the mesenteric artery, and every 15 minutes we stimulated at 8 Hz for 1 minute. Each stimulus was 10 V and 1 millisecond. The perfusate through the mesenteric vascular preparation was collected for measurement of norepinephrine (NE) by high-performance liquid chromatography. NE overflow was defined as NE content of perfusates per wet mesenteric artery weight. To identify component of NE overflow that was affected by Mg2+, we applied cocaine, a specific blocker of NE reuptake by nerve tissue, or deoxycorticosterone, a blocker of NE uptake by non-neuronal tissue.

Protocol 3: Effect of Extracellular Mg2+ on Voltage-Gated Ca2+ Channels

The PC12 cells were cultured in Dulbecco modified eagle medium containing 10% fetal calf serum and 2.5% NGF (10 ng/mL) for 7 days. We selected cells with neurite outgrowth for the electrophysiology experiments.

The perforated whole-cell clamp technique was used to avoid the run-down of voltage-gated Ca2+ currents. Ba2+ ion was used as a charge carrier through the voltage-gated Ca2+ channels. The standard patch electrode solution contained 1.2 mmol/L of Mg2+. Low-Mg2+ and the high-Mg2+ extracellular solutions were prepared as described in protocol 2. Extracellular solutions containing different concentrations of Mg2+ were applied by changing the perfusion solution. All the data were corrected for the liquid junction potential (−4 to −2 mV). Amphotericin B (200 ng/mL) was used for the perforated whole-cell clamp experiments.

An L-type Ca2+ channel blocker, nitrendipine (NIT) (5 μmol/L), and an N-type Ca2+ channel blocker, ω-conotoxin GVIA (ω-CgTX) (1 μmol/L), were used to identify the type of voltage-gated Ca2+ current.

Statistical Analysis

In protocol 2, the average of the control responses of NE overflow to electrical stimulation and the following 2 responses in the different Mg2+ concentration buffers or in the presence of drugs were calculated. The results are expressed as total NE overflow standardized by tissue weight and the percent change from the averaged NE overflow in control buffer.

Data are shown as means±SEM in protocols 1 and 2 and as means±SD in protocol 3. The statistical analysis in protocol 2 was performed by the paired and unpaired Student t test, and in protocols 1 and 3 by analysis of variance (ANOVA). P<0.05 was considered indicative of statistical significance.

Results

Protocol 1: Sympatholytic Effect of Mg2+ and Blood Pressure Changes In Vivo

The body weight of the rats infused with Mg2+ (Mg2+ group) was 241±2 g, their baseline MAP was 175±5 mm Hg, and their HR was 453±9 bpm, which were not affected by the Mg2+ infusion (MAP, 170±4 mm Hg; HR, 440±7 bpm). The serum Mg level significantly increased from 2.5±0.05 mg/dL to 3.5±0.11 mg/dL after Mg2+ infusion (P<0.01). The body weight of the rats in the control group was 245±4 g, their baseline MAP was 173±3 mm Hg, and their HR was 442±12 bpm. None of those parameters changed much after saline infusion in the control group (MAP, 174±5 mm Hg; HR, 444±10 bpm; serum magnesium from 2.5±0.05 mg/dL to 2.5±0.06 mg/dL). Hydralazine decreased MAP dose-dependently and the decrease was more prominent in the Mg2+ group than control group (Figure 1a), whereas HR increased more in the control group than in the Mg2+ group (Figure 1b).

Blood Pressure Response to Electrical Stimulation in Pithed Sprague–Dawley Rats

The body weight of the rats infused with Mg2+ (Mg2+ group) was 271±10 g, their baseline MAP after pithing was 55±11 mm Hg, and their HR was 206±25 bpm. The body weight of the rats in the control group was 275±16 g, their baseline MAP after pithing was 53±10 mm Hg, and their HR was 208±22 bpm. Electrical stimulation significantly increased both MAP and HR, but the extent of the increase was greater in the control group than in the Mg2+ group (Figure 2).

Figure 1. BP and heart rate (HR) responses to hydralazine in control and Mg2+ infused groups. a, Decreases in MAP of the Mg2+ group (●) were significantly larger than those of the control group (□). b, Increases in HR of the Mg2+ group (●) were significantly smaller than those of the control group (□). Data are presented as means±SEM, P<0.01 by ANOVA and Sheffe method.
The pressor responses to electrical stimulation were completely abolished (n=3; MAP, 55±8 to 53±4 mm Hg; HR, 205±10 to 204±8 bpm) by 1 mg/kg hexamethonium, suggesting that the pressor response reflects peripheral sympathetic stimulation.

Protocol 2: Effect of Mg²⁺ on NE Release From Peripheral Sympathetic Nerve Endings

NE overflow at 1.2 mmol/L Mg²⁺ was 0.266±0.032 ng/g tissue weight, and percent change of NE overflow with each buffer is shown in the Table. High-Mg²⁺ buffer significantly suppressed NE overflow.

In the presence of cocaine or deoxycorticosterone, the high-Mg²⁺ buffer significantly attenuated NE overflow, and the Mg²⁺-induced changes were not significantly altered.

Protocol 3: Effect of Extracellular Mg²⁺ on the Voltage-Gated Ca²⁺ Currents

To determine the effect of extracellular Mg²⁺ on NE release, we investigated the effect of extracellular Mg²⁺ on the voltage-gated Ca²⁺ currents of PC12 cells differentiated by NGF. Figure 3A shows the Ba²⁺ currents through voltage-gated Ca²⁺ channels recorded under the voltage clamp from a PC12 cell at 3 different extracellular Mg²⁺ concentrations. The holding potential was −74 mV, and a test pulse step to 6 mV was applied. The Ba²⁺ current exhibited a clear inactivation process, but the steady current persisted. The Ba²⁺ current first appeared at a potential step to −34 mV. As the depolarizing steps became greater, the amplitude of the Ba²⁺ current increased. When the extracellular Mg²⁺ concentration was changed sequentially, the amplitude of the peak current was largest in the 0.3 mmol/L Mg²⁺ solution, smaller in the standard extracellular solution (1.2 mmol/L Mg), and smallest in the 4.8 mmol/L Mg²⁺ solution. Increases in the extracellular Mg²⁺ concentration inhibited the voltage-gated Ca²⁺ current, and the inhibition was reversible when the extracellular solution was changed to the 0.3 mmol/L Mg²⁺ solution (0.3 mmol/L Mg²⁺ recover).

The current–potential relationships of the Ba²⁺ current in the extracellular solutions containing 3 different Mg²⁺ concentrations are shown in Figure 3B. Increased extracellular Mg²⁺ induced a decrease in amplitude of the Ba²⁺ current at all potentials, indicating that inhibition of the Ba²⁺ current was not voltage-dependent. Figure 3C summarizes the amplitude of the Ba²⁺ current at the 3 Mg²⁺ concentrations. The amplitude of the peak Ba²⁺ current in the standard extracellular solution was normalized as 100% in each record. Extracellular Mg²⁺ inhibited the Ba²⁺ current in a concentration-dependent manner when analyzed by repeated measures ANOVA with post-test for linear trend. We could observe significant linear trend (P<0.0001).

Figure 4A shows the sequential effect of NIT (5 μmol/L) and 4.8 mmol/L Mg²⁺ on the Ba²⁺ current. Application of NIT decreased the current by ≈17% of the control, and

<table>
<thead>
<tr>
<th>Percent Change of Norepinephrine Overflow From Peripheral Nerve Endings</th>
<th>Control</th>
<th>Cocaine</th>
<th>Deoxycorticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Mg²⁺ (0.3 mmol/L Mg²⁺)</td>
<td>113±5% (n=10)</td>
<td>115±8% (n=5)</td>
<td>108±7% (n=5)</td>
</tr>
<tr>
<td>High Mg²⁺ (4.8 mmol/L Mg²⁺)</td>
<td>51±2% (n=10)*</td>
<td>53±4% (n=5)*</td>
<td>49±5% (n=5)*</td>
</tr>
</tbody>
</table>

Norepinephrine overflows were calculated as percent changes from 1.2 mmol/L Mg²⁺ condition. Data are presented as means±SEM. *P<0.01 compared to 1.2 mmol/L Mg²⁺ buffer by paired t test.
Figure 3. Effect of extracellular Mg$^{2+}$ on voltage-gated Ca$^{2+}$ current. Currents were recorded under voltage-clamp with the perforated whole-cell clamp technique from a PC12 cell differentiated by NGF. A, Ba$^{2+}$ currents were evoked by a pulse step to 6 mV from the holding potential of −74 mV in extracellular solutions containing 0.3 mmol/L Mg$^{2+}$, 1.2 mmol/L Mg$^{2+}$, 4.8 mmol/L Mg$^{2+}$, and again in 0.3 mmol/L, Mg$^{2+}$ to examine the recovery. B, The current–potential relationship of the Ba$^{2+}$ current in each Mg$^{2+}$ level (●, 0.3 mmol/L; ○, 1.2 mmol/L; △, 4.8 mmol/L) are plotted. The ordinate indicates the peak amplitude of the Ba$^{2+}$ current in pA and the abscissa indicates test potential in mV. C, Concentration dependency of the effect of Mg$^{2+}$ on the Ba$^{2+}$ current. The amplitude of the peak Ba$^{2+}$ current in the 1.2 mmol/L Mg$^{2+}$ solution was normalized as 100% in each record. The mean amplitude of the current in 1.2 mmol/L Mg$^{2+}$ solution was 124 ± 44 pA. Means and standard deviations of the currents in 0.3 mmol/L, 3.6 mmol/L, 2.4 mmol/L, and 4.8 mmol/L Mg$^{2+}$ normalized to that in 1.2 mmol/L Mg$^{2+}$ were 124 ± 19%, 83 ± 10%, 75 ± 7%, and 68 ± 15%, respectively. *Statistical significance (P<0.05) using 1-way ANOVA with Tukey multiple comparison test.

Figure 4. Inhibition of N-type Ca$^{2+}$ current by high Mg$^{2+}$. A, Effect of L-type Ca$^{2+}$ channel blocker, nifedipine (NIT), and high Mg$^{2+}$ on the Ba$^{2+}$ current. The Ba$^{2+}$ current was evoked by the pulse step to 6 mV from the holding potential of −74 mV. The amplitude of the peak current was measured every 10 seconds. The ordinate indicates percentage of the Ba$^{2+}$ current as compared with the control (before application of channel blocker in 1.2 mmol/L Mg$^{2+}$) and the abscissa the time course of the application of 5 μmol/L NIT and 4.8 mmol/L Mg$^{2+}$. After NIT attained its maximal effect, 4.8 mmol/L Mg$^{2+}$ solution with NIT was applied. B, Effect of N-type Ca$^{2+}$ channel blocker, ω-conotoxin GVIA (CgTX), and 4.8 mmol/L Mg$^{2+}$ on the Ba$^{2+}$ current. The Ba$^{2+}$ current was recorded by the same method as in (A). The ordinate indicates percentage of the Ba$^{2+}$ current as compared with the control (before application of CgTX in 1.2 mmol/L Mg$^{2+}$) and the abscissa, and the time course of the application of 1 μmol/L CgTX and 4.8 mmol/L Mg$^{2+}$. After CgTX attained its maximal effect, 4.8 mmol/L Mg$^{2+}$ solution with CgTX was applied. C, Summary of the experiments. The ordinate indicates the mean and standard deviation of the normalized Ba$^{2+}$ current as compared with the control (before application of channel blocker in 1.2 mmol/L Mg$^{2+}$). High Mg$^{2+}$ indicates application of 4.8 mmol/L Mg$^{2+}$ solution.
the majority of the remaining current was L-type Ca\(^{2+}\) channels, and that both of these currents are inhibited by high Mg\(^{2+}\).

**Discussion**

Mg\(^{2+}\) is reported to modulate cardiac function and vasomotor control by nonspecific antagonism of the Ca\(^{2+}\) channels.\(^{4,5,7,19-21}\) Mg\(^{2+}\) also modulates neuronal activity\(^6\) but the precise mechanisms are still unknown. In the present study, we showed an Mg\(^{2+}\) effect on sympathetic tone both in vivo and in vitro studies. Firstly, using both conscious spontaneously hypertensive rats and pithed rats, we showed that Mg\(^{2+}\) possesses sympatholytic effects and reduces blood pressure. Secondary, using perfused mesenteric artery preparation, we revealed that Mg\(^{2+}\) reduces norepinephrine release. Finally, Mg\(^{2+}\) inhibits N-type Ca\(^{2+}\) channels in differentiated PC12 cells. The results of the present study indicate that Mg\(^{2+}\) attenuated sympathetic tone mainly by inhibiting N-type Ca\(^{2+}\) channels.

Mg\(^{2+}\) augmented the antihypertensive effect of hydralazine and attenuated the reflex tachycardia. It suggests that Mg\(^{2+}\) exerts an inhibitory effect on sympathetic tone and enhances the vasodilator effect of hydralazine. To further investigate roles of peripheral sympathetic nerves on blood pressure regulation, we applied the mechanically pithed preparation in which central nervous activities are fully destroyed and also we can avoid pharmacological interference. Electrical stimuli were applied at the level of 7th to 10th thoracic vertebrae to direct stimulation of cardiac sympathetic nerve be possible.\(^{14}\) Mg\(^{2+}\) attenuated blood pressure elevation evoked by sympathetic nerve stimulation. Norepinephrine is a main factor that is released by electrical stimulation and regulates blood pressure in the pithed model; however, other circulatory factors could be released. Thus, we applied ex vivo model to investigate effect of Mg\(^{2+}\) on norepinephrine release. We measured NE overflow from the periarterial plexus of the mesenteric artery. There are 2 components to NE overflow: NE release from nerve endings and NE reuptake into nerve terminals and other tissue. We showed that Mg\(^{2+}\) affects NE release but not uptake by using uptake blockers such as cocaine\(^{16}\) and deoxycorticosterone.\(^{17}\)

To specify Mg\(^{2+}\) effect on Ca\(^{2+}\) channels that play a pivotal role in sympathetic activity, we used NGF-treated PC12 cells. NGF-treated PC12 cells exhibited neurite outgrowth. Approximately 90% of the voltage-gated Ca\(^{2+}\) channel currents were composed of \(\omega\)-CgTX-sensitive N-type Ca\(^{2+}\) currents. An L-type Ca\(^{2+}\) channel blocker, NIT, reduced Ca\(^{2+}\) influx to a smaller extent than \(\omega\)-CgTX. These characteristics are consistent with those of differentiated PC12 cells, which have characteristics similar to those of noradrenergic sympathetic neurons, including the development of N-type Ca\(^{2+}\) channels.\(^{12,13,22}\) In the present study, Mg\(^{2+}\) further inhibited the Ba\(^{2+}\) current after treatment with NIT but not after \(\omega\)-CgTX, suggesting that Mg\(^{2+}\) inhibits N-type Ca\(^{2+}\) channels. The high Mg\(^{2+}\)-induced inhibition of N-type Ca\(^{2+}\) channels observed in the present study was similar to the effect of proadrenomedullin N-terminal 20 peptide on the N-type current in NGF-treated PC 12 cells.\(^{18}\) This inhibition of the N-type Ca\(^{2+}\) current plays a role in the inhibition of Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels and as a result reduces the intracellular Ca\(^{2+}\) concentration.

Based on these results, extracellular Mg\(^{2+}\) blocks N-type and partly L-type Ca\(^{2+}\) channels, and thus inhibits NE release, and these effects play an important role in regulating sympathetic tone and blood pressure.

**Perspectives**

Recent large clinical studies have shown that the lower the blood pressure obtained by treatment, the more successful in preventing hypertensive patients from cardiovascular events.\(^{23-25}\) Although dihydropyridine Ca\(^{2+}\) channel blockers are potent antihypertensive agents, short-acting Ca\(^{2+}\) channel blockers have been shown to be unfavorable for the treatment of hypertension.\(^{26}\) This may be partly caused by reflex sympathoactivation and catecholamine release, which induce unfavorable effects.\(^{8,9}\) Several epidemiological studies have supported tachycardia, an indicator of sympathetic tone, as an independent risk factor for cardiovascular death in elderly men.\(^{27}\) Because all vasodilators induce reflex sympathoactivation in hypertensive patients, treatment that aims to reduce sympathoactivation can achieve a greater therapeutic effect. Because Mg\(^{2+}\) decreases NE release from nerve endings by inhibiting voltage-gated N-type Ca\(^{2+}\) currents, Mg\(^{2+}\) may be beneficial in preventing organ damage by inhibiting reflex sympathoactivation. In fact, some studies have shown that Mg\(^{2+}\) supplementation improves the outcome of cardiovascular diseases.\(^{11,28}\) However, the antihypertensive action of Mg\(^{2+}\) is very weak.\(^{29}\) It led us to the speculation that Ca\(^{2+}\)
channel blockers with the inhibitory action of both an l-type and N-type Ca\(^{2+}\) channels such as cilnidipine\(^{30,32}\) might be efficacious for the treatment of hypertension.

References
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