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Inhibition of L-type calcium currents by magnesium sulfate on the rat basilar artery smooth muscle cells

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Abstract

Objective: Vasospasm remains the leading cause of cerebral damage after aneurysmal subarachnoid hemorrhage. Although magnesium regulates the calcium influx in vascular smooth muscle and endothelial cells, it has not been reported whether L-type calcium channels are involved in magnesium-induced vascular relaxation in rat basilar artery. So, the effect of magnesium sulfate on L-type calcium currents in freshly isolated smooth muscle cells from rat basilar artery was investigated. *Methods:* The smooth muscle cells were isolated from rabbit basilar artery by enzyme treatment. L-type Ca^{2+} currents were identified using cesium chloride, a potassium channel blocker and Bay K8644, an activator of L-type Ca^{2+} channel. Currents were recorded under step pulse whole cell patch clamp technique. *Results:* In the presence of cesium chloride (in pipette solution), inward currents were observed by depolarizing step pulses. The inward currents were significantly reduced by nimodipine (n=4, $p<0.05$), an L-type Ca^{2+} channel blocker and increased by Bay K8644 (n=5, $p<0.05$), an L-type Ca^{2+} channel activator. The L-type calcium currents (156±17.0 pA, n=12) were significantly reduced by the application of 5 mM magnesium sulfate (53.8±7.0 pA, n=12, $p<0.01$).
Conclusion: These results suggest that magnesium may relax cerebral vessel of rat basilar artery through decreasing intracellular Ca^{2+} ion by inhibition of L-type Ca^{2+} channels.

INTRODUCTION

Cerebral vasospasm most frequently occurs between the fourth and tenth day after subarachnoid hemorrhage (SAH) from the ruptured cerebral aneurysm. It is one of the major cause of cerebral ischemia and leading cause of death and disability following aneurysm rupture. The factors that can increase the risk of cerebral vasospasm include prolongation of the subarachnoid clot by antifibrinolytic drugs, hypotension, inappropriate treatment of hyponatremia, hypovolemia, hyperthermia and increased intracranial pressure.[1,2] However the pathogenesis of narrowing of central lumen is not completely understood and the way of treatment is not yet clear. The adjunctive therapies have been developing based on etiology of vasospasm at cellular levels. Magnesium is one of the adjuncts being used.[3] Moreover, experimental and clinical studies have demonstrated that hypomagnesaemia causes an elevation of intracellular calcium concentration and thus vascular smooth muscle contraction. Moreover, experimental and clinical studies have demonstrated the beneficial effects and safety of using magnesium in SAH.[3-7] Magnesium is well known vasodilator and is one of the most abundant cations in living cells playing roles in various cellular processes. Magnesium shares the similar properties as that of calcium ion channel antagonists and considered as physiological calcium channel blocker.[8] In addition, magnesium sulfate acts as a vasodilator by increasing the synthesis of prostacyclin, as well as inhibiting angiotensin converting enzyme activity.[9] Although magnesium sulfate has been used clinically in the management of cerebral vasospasm, the action mechanism of vasodilation by magnesium sulfate has not been fully understood. Therefore, this study was conducted to clarify and confirm the effect of magnesium on L-type calcium currents in

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freshly isolated cerebral smooth muscle cells from rat basilar artery using patch clamp technique.

METHODS

Cell isolation

Rat basilar smooth muscle cells were isolated as previously described.[10,11] Briefly, Sprague-Dawley male rats (200-300 g) were anesthetized with diethyl ether and decapitated. The basilar artery was removed to a medium containing NaCl (130 mM), KCl (5 mM), $CaCl_2$ (0.8 mM), $MgCl_2$ (1.3 mM), glucose (5 mM), N[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid] (HEPES, 10 mM), penicillin (100 units/ml) and streptomycin (0.1 g/l). The basilar artery was then cleaned from the connective tissue and the small side branches. The arteries were cut in 2.0 mm rings and incubated for 1 hour at room temperature in a medium containing $CaCl_2$ (0.2 mM), collagenase (type II, 0.5 g/l), elastase (0.5 g/l), and hyluronidase (type IV-S, 0.5 g/l). The rings were washed in fresh solution containing $CaCl_2$ (0.2 mM), trypsin inhibitor (0.5 g/l), and then triturated gently. Cells were placed on glass coverslips and stored at 4 C^o in the same medium containing $CaCl_2$ (0.8 mM) and bovine serum albumin (2 g/l) free of essential fatty acids. The cells were used within 12 hours after isolation.

Whole-cell current recording

As previously described[12,13], cells were voltage clamped using the whole-cell patch clamp technique. Patch pipettes were made from glass capillary (PG10165-4, WPI Inc.USA) using patch electrode puller (PC10, Narishige, Tokyo, Japan). The patch pipette resistance ranged 2-10 MΩ. The pipettes were positioned using a three dimensional vernier-type hydraulic micromanipulator (MX-630R, SOMA SCIENTIFIC). Giga-Ohm seals were formed by applying gentle negative pressure. Voltage steps were applied with pulse protocol driven by an IBM computer equipped with A-D and D-A converter (Digidata 1200, Axon Instrument Inc., Foster city, CA, USA). Membrane currents were recorded and amplified using Axon-patch 200B and Clampex7 program (Axon instruments). Signals were filtered with a low-pass Bessel Filter (-2 dB at 1 kHz) and digitized online at sampling frequency of 5-10 kHz for subsequent computer analysis. Data analysis was performed using Clampfit 9. All experiments were carried out at room temperature.

Solutions and drugs

The seals were made in bath solution containing (mM) NaCl 125, KCl 5, $MgCl_2$ 1, $BaCl_2$ 10, HEPES 10, glucose 12.5 (pH was adjusted to 7.4 with NaOH). To record L-type Ca^{2+}channel currents, bath solution was changed to solution containing (mM) tetraethylammonium chloride (TEA-Cl) 125, 4-aminopyridine 5, $MgCl_2$ 1, $BaCl_2$ 10, HEPES 10, glucose 12.5 (pH was adjusted to 7.2 with TEAOH). Pipette solution (mM) was composed of CsCl 135, $MgCl_2$ 4,$CaCl_2$ 1, ethylene glycol-bis (b-aminoethyl-ether)N,N,N',N'-tetra acetic acid (EGTA) 11, HEPES 10, Na_2-ATP 2 and GTP 0.5 (pH was adjusted to 7.2 with CsOH).

Statistical analysis

All values were expressed as mean±SEM. Student's t-test was used to compare the means between experimental groups. One-way analysis of variance (ANOVA) was performed to compare the means between more than two groups. $P<0.05$ was considered to be statistically significant.

RESULTS

In order to clarify the role of L-type calcium channels on the magnesium-induced vascular relaxation, we examined the effect of 5 mM magnesium sulfate on L-type calcium currents in rat basilar artery smooth muscle cells. To maximize the Ca^{2+} currents, 10 mM Ba^{2+} solution was used. All experiments were performed within 30 minutes since Ba^{2+} remained stable for 30 minutes.[14] The inward currents evoked by step pulses (from -50 to + 70 mV in 10 mV increment from a holding potential of -60 mV) showed voltage dependency. Figure 1A shows the current traces by step pulses. The activation threshold of inward current was around -30 mV and the maximum inward currents were shown around +10 mV (Fig 1B). Further, to check whether the inward currents were mediated by L-type Ca^{2+} channels, we applied nimodipine, an L-type Ca^{2+} channel blocker and Bay K8644, an L-type Ca^{2+} channel activator respectively. As shown in Figure 2A, the step pulse-induced inward currents (control: 148.8±31 pA, n=4) were suppressed by nimodipine (1 μM, 55±7 pA, n=4, $p<0.01$) and enhanced by Bay K8644 (1 μM, 291±50 pA, n=5, $p<0.05$). Figure 2B shows the relative currents by Bay K8644 and nimodipine. These results indicate that the step pulse-induced inward currents are mediated by L-type Ca^{2+} channel activation.

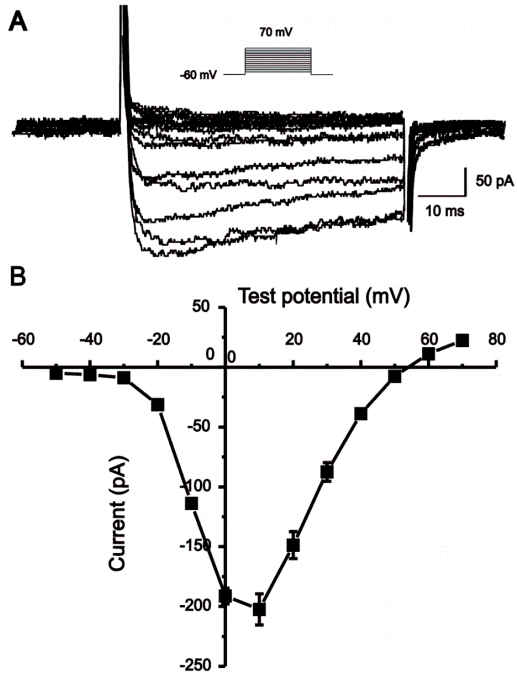

Figure 1 (A). Representative current traces by step pulses from -50 to +70 mV at 10 mV interval at a holding potential of -60 mV. The peak current was detected at +10 mV. (B). Current-voltage relationships from 12 cells recorded.

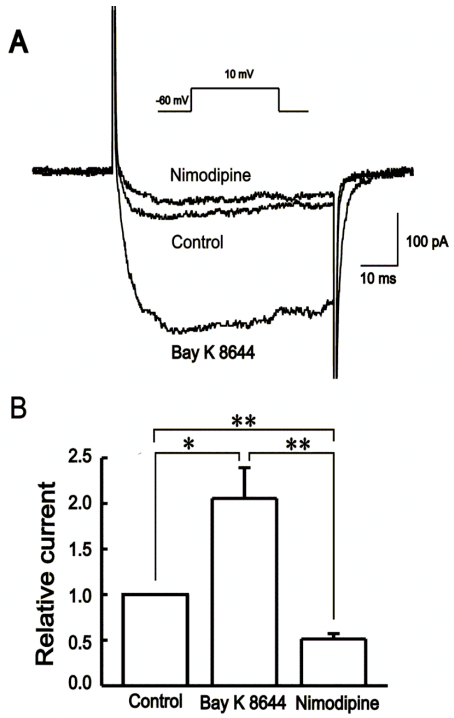

Figure 2 (A). Current traces showing the suppression of inward control current (148.8±31, pA n=4) by nimodipine (1 μM), an L-type calcium channel blocker (55±7 pA, n=4, $p<0.01$, One-way ANOVA, post hoc Scheffé test) and the enhancement of inward current by Bay K8644 (1 μM, 291±50 pA, n=5, $p<0.05$, One-way ANOVA, post hoc Scheffé test) an L-type calcium channel activator. (B) Relative currents by Bay K8644 and nimodipine. *, ** represent $p<0.05$ and $p<0.01$, respectively.

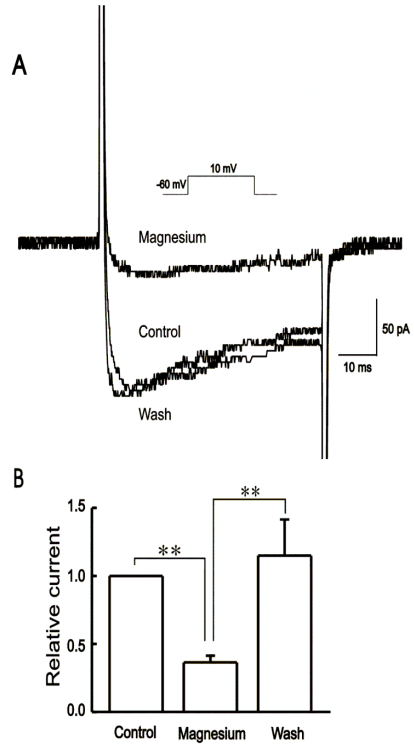

Figure 3. Effect of magnesium on L-type calcium current. (A) Current traces showing the suppression of L-type Ca^{2+} (control, 155±61 pA, n=12) current by magnesium application (5 mM) (53±7 pA, n=12, p<0.01, One-way ANOVA, post hoc Scheffé test). (B) Relative currents by magnesium and washout. ** represents p<0.01

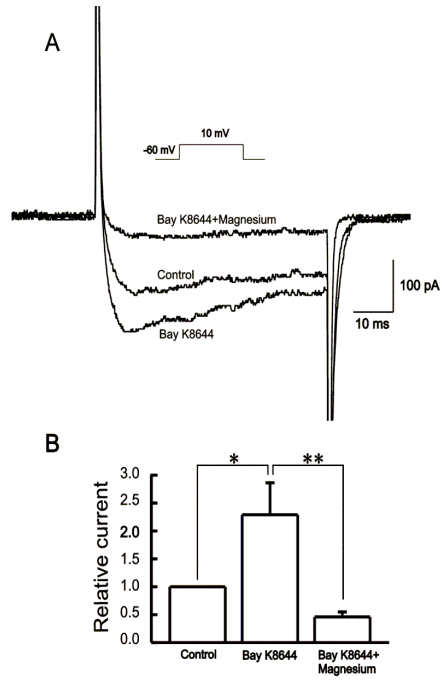

Figure 4 (A). Current traces showing the enhancement of L-type Ca^{2+} current by Bay K8644 (300±40 pA, n=4) and suppression of the Bay K8644-induced L type calcium currents by magnesium (66±14 pA, n=4; P<0.01, One-way ANOVA, post hoc Scheffé test). (B) Relative currents by Bay K8644 and magnesium in the presence of Bay K8644. * represents p<0.05, ** represents p<0.01.

To investigate the effect of magnesium on the L-type Ca^{2+} currents, we applied the bath solution including 5 mM magnesium sulfate**.** Figure 3A shows representative current traces induced by step pulse to +10 mV at the holding potential of -60 mV. The L-type calcium currents (Control, 155±61 pA, n=12) were significantly reduced by 5 mM magnesium sulfate (53±7 pA, n=12, $p<0.01$, Fig 3A) and recovered to control level by washout (192±61 pA, n=4). As shown in Figure 3B, the relative inward currents were suppressed by magnesium sulfate and recovered to control level after washout. These data indicate that magnesium reversibly suppresses the L-type Ca^{2+} currents on rat basilar artery smooth muscle cells. Further to confirm the blocking effect of magnesium, we applied magnesium sulfate in the presence of Bay K8644. As we anticipated, magnesium sulfate suppressed the Bay K8644-induced inward currents (Bay K8644: 300±40 pA, n=4 to 66±14 pA, n=4; $p<0.01$; Figure 4A). Figure 4B shows the relative currents by Bay K8644 and Bay K8644 in the presence of magnesium sulfate.

DISCUSSION

The intracellular free calcium level ($[Ca^{2+}]i$) plays a pivotal role in the regulation of smooth muscle contractility.[15] In the present study, our results provide one of the mechanism for magnesium induced vascular relaxation by inhibiting L-type calcium channel. L-type Ca^{2+} channels play an active role in regulation of smooth muscle tone. We have obtained a similar Ca^{2+}current which resembles with L-type Ca^{2+} current reported by others in cerebral smooth muscle cells.[2,10] To maximize the Ca^{2+} currents, 10 mM Ba^{2+} solution was used in the bath instead of Ca^{2+} solution because Ca^{2+} currents have been reported to be inactivated by an increase in $[Ca^{2+}]i$ in smooth muscle cells.[10,14,16] In this study, in the presence of a potassium channel blocker (cesium chloride) in pipette solution, we found that the voltage step induced inward currents were significantly reduced by nimodipine, and increased by Bay K8644, with significant reduction by the application of 5 mM magnesium sulfate. These results are in good agreement with our previous study in rabbit basilar artery smooth muscle cells.[17] In addition, other studies have also reported that magnesium decreases the contraction of the detrusor muscle of the bladder by inhibiting L-type Ca^{2+} channels.[18] The cause of vasospasm, whether angiographic or clinical, is still a source of debate. The inflammatory response to SAH does appear to contribute to the etiology of cerebral vasospasm, specifically the role of leukocytes in releasing oxyhemoglobin after lysing extravasated erythrocytes in the subarachnoid space.[19,20] Peterson *et al*.[21] found that RBC lysis was necessary for the development of vasospasm after the subarachnoid injection into the dog. Erythrocyte lysate increases intracellular Ca^{2+} ($[Ca^{2+}]i$), contracts cerebral arteries and has been suggested to be the causative agent for cerebral vasospasm. However, the mechanism of erythrocyte lysate-induced $[Ca^{2+}]i$ mobilization is not clear. Kim CJ *et al*. demonstrated that the erythrocyte lysate releases Ca^{2+} from IP3-sensitive intracellular stores and produces Ca^{2+} entry from voltage-independent Ca^{2+} pathways.[22]

A disruption of the ability of smooth muscle cells to regulate calcium after SAH has also been proposed[10], although the contractile apparatus may be more or less sensitive to calcium following SAH.[23,24] Most treatment protocols for vasospasm include triple-H therapy such as hemodynamic therapy and nimodipine, a calcium channel blocker.[25,26] Influx of extracellular calcium into vascular smooth muscle plays a critical role in the development of vasospasm. Voltage dependent Ca^{2+} channel have been investigated in smooth muscle cells including cerebral vessels.[5,27-31] The sustained influx of calcium in vascular smooth muscle results in activation of various second messenger systems that permute contractility.[3] Ca^{2+} influx pathway especially L-type Ca^{2+} channels in cerebral smooth muscles, have been investigated using patch clamp techniques. L-type Ca^{2+} channels have been characterized in rabbit, guinea pig and rat basilar arteries smooth muscle cells using whole cell recording and play an active role in regulation of smooth muscle tone.[17,31-35] Magnesium is not only the physiologically most abundant free divalent cation, but also it exerts important direct effects on certain ion channels. External magnesium (1-5 mM) has been shown to produce discrete blocking events on unitary inward currents carried by Ba^{2+}, and therefore has been implicated as a pore blocker.[36] The exact mechanism of Mg^{2+} on vascular smooth muscle is still under debate, but the antagonist relationship of calcium and magnesium may play some important role. Magnesium-therapy has already been demonstrated to be both safe and effective therapy in preventing neurological complications in obstetrics patients with eclampsia.[3]

Magnesium has similar biochemical profile like other L-type Ca^{2+} channel blockers. Zhang *et al*. demonstrated that magnesium is similar

to nifedipine in that, it was shown to reduce the depolarization of smooth muscle membranes[37] and L-type Ca^{2+} channels have binding site for magnesium within the C-terminal region of the α-subunit.[8,9] Consistent with our previous patch clamp study on rabbit basilar artery, we demonstrated that 5 mM magnesium significantly inhibits the L-type calcium current on rat basilar artery.[17] The electrophysiological and pharmacological characteristics of Ca^{2+} current in this study are consistent with the results.

Calcium channel blockers including nimodipine inhibit the calcium influx through L-type calcium channel on smooth muscle cells, and it has been used in prevention of vasospasm.[9,38] For instance, magnesium reduces the depolarization of smooth muscle membrane and serotonin-induced calcium influx was reversed by the addition of magnesium.[37,38] Moreover, the study by Farago *et al*.[39] in 1991, supports a role for Mg^{2+} deficiency in the development of the cerebral vasospasm. Clinical trials by Yahia *et al*.[40] in 2005 confirmed the safety and feasibility of a continuous infusion of intravenous $MgSO_4$ in patients with aneurysmal SAH. In addition, intravenous magnesium can reduce cerebral ischemic events after aneurysmal subarachnoid hemorrhage by attenuating vasospasm and increasing the ischemic tolerance during critical hypoperfusion.[41] Numerous experimental stroke models have demonstrated that magnesium is neuroprotective. In experimental SAH models, magnesium sulfate reverses cerebral vasospasm and infarct volume.[6,42,43]

In this study, authors can conclude that magnesium in concentration of 5 mM decreases the L-type Ca^{2+} currents in rat basilar artery smooth muscle cells. Decrease in Ca^{2+} entry via inhibition of L-type Ca^{2+} channels explains some of the mechanism for the magnesium induced-vascular relaxation and provide background for clinical use of magnesium sulfate, especially in treating cerebral vasospasm following SAH.

ACKNOWLEDGEMENTS

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