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Magnesium lithospermate B decreases $[Ca^{2+}]i$ in endothelial cells by inhibiting K^+ currents

Haifei Zhang, Jie Zhang, Ruopeng Zha, Miaomiao Hu, Yiping Wang *

State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

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ABSTRACT

Magnesium lithospermate B (MLB) is a hydrophilic active component of *Salviae miltiorrhizae* Radix. Studies have shown that MLB affected intracellular calcium ($[Ca^{2+}]i$), but the underlying mechanism was unclear yet. The present work was aimed to investigate the underlying mechanism of MLB affecting $[Ca^{2+}]i$ in endothelial cells (ECs). Isolated mesentery arteries were employed to test the involvement of L-Ca^{2+} channel. $[Ca^{2+}]i$ was measured in ECs loaded with Fluo-3. Membrane potential and membrane currents were recorded in ECs using patch-clamp techniques. Results showed that MLB did not inhibit Ca^{2+} influx via L-Ca^{2+} channel in isolated mesenteric arteries. However, MLB decreased $[Ca^{2+}]i$ in a concentration-dependent manner in ECs. MLB depolarized the membrane potential of ECs and inhibited K^+ currents. These results demonstrated that MLB decreased $[Ca^{2+}]i$ by inhibiting K^+ currents and depolarizing membrane potential in ECs.

1. Introduction

The endothelium plays a key role in the regulation of the cardiovascular system. Endothelial cells (ECs) express a variety of ion channels. The most obvious function of these channels is to control two vital parameters: calcium (Ca^{2+}) influx and membrane potential. Intracellular Ca^{2+} ($[Ca^{2+}]i$) is critical for the regulation of ECs function. Ca^{2+} enters ECs via nonselective cation channels. Membrane potential modulates the electrochemical gradient for diffusion of Ca^{2+} into ECs with hyperpolarization augmenting Ca^{2+} influx and depolarization inhibiting Ca^{2+} influx (Adams et al., 1989; Ay et al., 2004; Campbell et al., 1991; Cannell and Sage, 1989).

Magnesium lithospermate B (MLB, Fig. 1) is a hydrophilic active component of *Salviae miltiorrhizae* Radix, a traditional Chinese herbal medicine used in the treatment of cardiovascular diseases. MLB has been used as an active marker of *S. miltiorrhizae* Radix products by the National Pharmacopoeia Council of China (Hu et al., 2005). MLB exerts free radical scavenging (Chen et al., 1999; Wu et al., 2000; Yokozawa et al., 1995) and antihypotensive effects (Kamata et al., 1994). It also inhibits platelet aggregation (Onitsuka et al., 1983), and protects HUVECs against homocysteine-induced endothelial dysfunction (Chan et al., 2004).

Recent studies demonstrated that pretreatment of the cells with MLB blocked H_2O_2-induced elevation in $[Ca^{2+}]i$ level and apoptotic death (Liu et al., 2007).

But up to the present time, it is still not clear whether MLB decreases $[Ca^{2+}]i$ by blocking calcium channels or through other mechanisms. In this study, we sought to investigate the underlying mechanism of MLB affecting $[Ca^{2+}]i$ in ECs.

2. Methods and materials

2.1. Materials

MLB (brown powder with 99.7% purity) was obtained from the Research Center of Traditional Chinese Medicine Modernization, Shanghai Institute of Materia Medica. EGTA, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), sodium deoxycholate, acetylcholine, and tetraethylammonium (TEA) were from Sigma-Aldrich, China, Inc. MCDB-131and Fluo-3AM were from Invitrogen. MLB was dissolved in Krebs for measurement of $[Ca^{2+}]i$ and currents recording, or in high K^+,Ca^{2+}-free Krebs for tone recording just before every experiment. Krebs contained (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 Hepes (pH = 7.4, titrated with NaOH). The components of Ca^{2+}-free Krebs were similar to those of Krebs but EGTA 0.2 mM was added instead of $CaCl_2$. High K^+,Ca^{2+}-free Krebs consisted of (in mM): NaCl 17, KCl 100, and other components were similar to those of Ca^{2+}-free Krebs. The pipette solution comprised (in mM): 100 K gluconate, 30 KCl, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 3 EGTA, 10 Hepes, and 10 glucose (pH = 7.2 titrated with KOH). 1 mg Fluo-3/AM was dissolved in DMSO

* Corresponding author. State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Shanghai 201203, China.
E-mail address: ypwang@mail.shcnc.ac.cn (Y. Wang).

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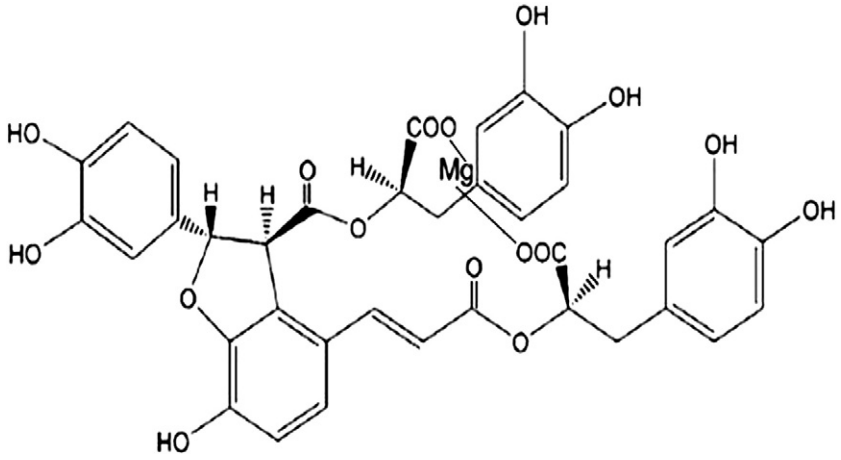

Fig. 1. Structure of magnesium lithospermate B.

with 20% Pluronic F-127 as 1 mM stock solution, stored in 4 μl aliquots at −20 °C.

2.2. Isolation of rat mesenteric arteries and tone recording

The animal experiments conformed with the institutional guidelines on the care and use of experimental animals approved by the Animal Care and Use Committee of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Male Sprague–Dawley rats (250–300 g, grade II) were killed by injecting a lethal dose (80 mg/kg) of sodium pentobarbitone. The superior mesenteric artery was carefully removed and put in iced Krebs. After the connective tissues were removed carefully, mesenteric arteries were cut into 1.5 mm length artery rings and mounted into 20 ml chambers of an integrated myograph system (AD Instrument PowerLab) for tone recording. To remove vascular endothelium, artery rings were perfused with 1.80 mg/ml sodium deoxycholate in saline for 30 s then rinsed with sodium deoxycholate-free Krebs for 40 min. Chemical removal of the endothelium was assessed by the lack of a relaxant response to 1 nM acetylcholine. The chambers were filled with Krebs at 37 °C, and aerated with 95% O_2/5% CO_2 to maintain pH at 7.4. Tension signals were relayed to a PowerLab 4 amplifier and saved in an IBM computer system (sampling rate 100 Hz).

Artery rings were equilibrated in Ca^{2+}-free Krebs for 30 min and washed three times at 15-min intervals, then changed to high K^+, Ca^{2+}-free Krebs for 15 min. Subsequently, $CaCl_2$ (0.125–4 mM) was applied at 5-min intervals to produce concentration–response curves. When maximum vasoconstriction was achieved, the rings were washed three times and equilibrated for 30 min, then incubated for 15 min with 200 μM MLB or 1 μM verapamil. Concentration–response curves to cumulative application of $CaCl_2$ were then reconstructed. The maximum vasoconstriction achieved in control was taken as 100%.

2.3. Cell culture

HMEC-1, a human microvascular endothelial cell line, was obtained from the Cell Bank of the Chinese Academy of Sciences. The HMEC-1 cells were maintained in MCDB-131 medium supplemented with 10% bovine calf serum, benzylpenicillin 100 kU/L, streptomycin sulfate 100 kU/L, Hepes 4.7 mM, sodium bicarbonate 44 mM, and incubated at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. Measurement of $[Ca^{2+}]i$

HMEC-1 cells were loaded with 25 μM Fluo-3 AM fluorescent probe in Krebs buffer containing 2.5 mM Ca^{2+} according to the manufacturer's protocol. Then cells were washed three times with Krebs buffer , allowed to deesterify for 30 min and transferred to a quartz cuvette in high K^+-Krebs (K^+ 30 mM) at 37 °C on a fluorescence spectrophotometer (HITACHI F-4500, Japan) with 506 nm excitation and 525 emission wavelengths. To test the effect of KCl on $[Ca^{2+}]i$, 30 and 60 mM KCl were applied to HMEC-1 cells in normal Krebs. Triton was used to break the cell membrane and get maximum fluorescence. EGTA was applied to get minimum fluorescence subsequently.

2.5. Electrophysiological experiment

Membrane potential was recorded from HMEC-1 cells using the whole-cell configuration of the patch-clamp technique in current-clamp mode with Axopatch-1D amplifier (Axon Instruments, USA). Patch pipettes were pulled using a P-97 microelectrode puller (Shutter Instruments, Co., USA) with a pipette tip resistance of 1–5 MΩ. HMEC-1 cells were put in a 3 ml chamber mounted on the stage of a microscope (Optiphot-2; Nikon, Japan), and perfused continuously with an external solution via a PBS-8 solution exchange system (ALA Scientific Instruments Inc., USA) at a rate of 3 ml/min. Electrical contact with the cytosol was established using nystatin. Nystatin was dissolved in dimethyl sulphoxide and the final nystatin concentration in the pipette solution was 200 mM. Experiments were conducted at 37 °C. MLB was applied to the preparation by bath perfusion system. Comparisons of endothelial potential before and after application of MLB were performed after continuous recordings were successfully established.

Whole-cell outward K^+ current were recorded using the conventional patch-clamp method (Hamill et al., 1981). NPPB (100 μM) was applied to block Cl^- channels. After gigaseal formation (seal resistance >1 GΩ), the membrane was ruptured with gentle suction to obtain whole-cell voltage-clamp configuration. Voltage command protocols were provided by the pClamp 6.0.4 software package (Axon Instruments, USA) via a DigiData-1200 interface. Capacitance compensation was routinely optimized and series resistance was compensated by 40–80%. Linear leaks were subtracted digitally online. Currents were filtered at 1 kHz, sampled at 3 kHz. The mean cell capacitance was 8.8 ± 0.5 pF ($n = 23$). Currents during the last 400 ms in each step of two or three voltage-clamp trials were sampled and averaged before analysis. Currents were normalized to cell capacitance to obtain the current densities. All recordings were initiated in 5 min after the establishment of the whole-cell configuration, allowing equilibration of the pipette solution with the cell interior. Experiments were performed within 40 min after gaining whole-cell configuration. During this time, the amplitude of K^+ currents remained stable.

2.6. Statistical analysis

Vasocontraction was measured in grams and expressed as means ± S.E.M. The concentration of Ca^{2+} was calculated as: $C = K_d * (F - Fmin)/(Fmax - F)$, $K_d = 385$ nm, and expressed as means ± S.E.M.

Patch-clamp data was presented as means ± S.E.M. Data analysis was performed using the software Clampfit 9.0 (Axon Instruments, USA). Paired student *t*-test or one-way ANOVA was used, followed by Bonferroni post hoc test as appropriate. All tests were two-tailed and significance was set at $P \leq 0.05$.

3. Results

3.1. MLB did not inhibit vasoconstriction induced by Ca^{2+} influx

These studies were performed in high K^+,Ca^{2+}-free solution. KCl (100 mM) produced negligible increases in artery rings and subsequent stepwise application of $CaCl_2$ (0.125–4 mM) caused stepwise increases in the artery rings' tone. In denuded artery rings, 200 μM of MLB did not reduce the maximum contraction induced by $CaCl_2$ (93.78 ± 8.92%, $P > 0.05$) while 1 μM of verapamil reduced the maximum contraction to 17.6 ± 8.9% ($P < 0.001$), suggesting that MLB did not inhibit Ca^{2+} influx via L-Ca^{2+} channels. The concentration–response relationship was plotted as shown in Fig. 2.

3.2. MLB decreased intracellular Ca^{2+} in HMEC-1 cells

Stepwise application of KCl (30, 60 mM) induced decreasing of $[Ca^{2+}]i$ in HMEC-1 cells in normal Krebs (Fig. 3A). After HMEC-1 cells were suspended in modified-Krebs with KCl (30 mM) for 5 min, MLB (50, 100, 200 μM) was applied cumulatively. MLB decreased $[Ca^{2+}]i$ in a concentration-dependent manner with inhibitory percentage of 2.33%, 8.65% and 17.80%, respectively (Fig. 3).

3.3. MLB depolarized HMEC-1 cells membrane potential

Membrane potentials were recorded in current-clamp mode. HMEC-1 cells were perfused in high K^+ Krebs (30 mM). Different concentrations of MLB (50, 100, and 200 μM) were applied separately and membrane potentials were depolarized by 3.9%, 13.4% and 25.4%, respectively ($n = 8$). The resting membrane potential was −32.3 ±

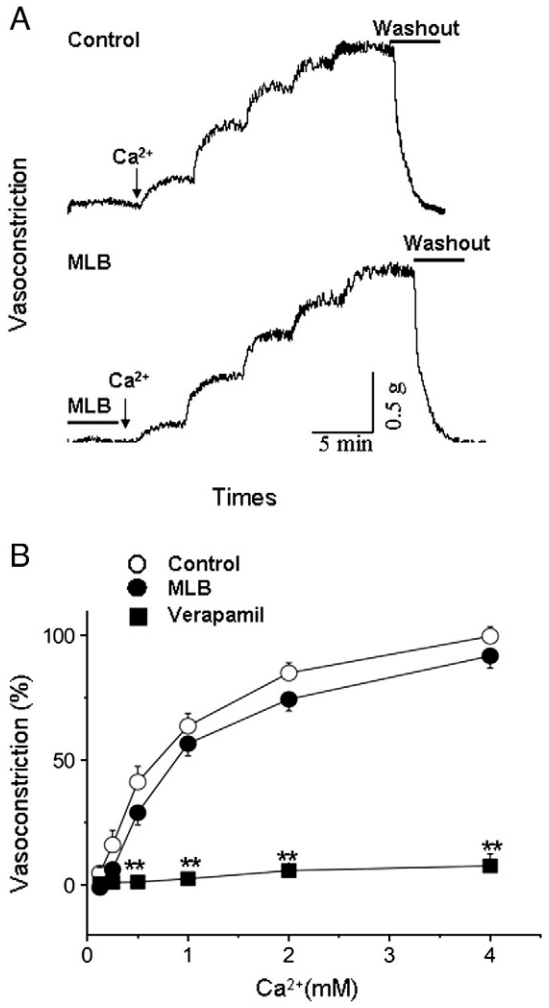

Fig. 2. MLB did not inhibit Ca^{2+} influx into smooth muscle cells. (A) MLB did not inhibit the artery ring contractions by stepwise application of Ca^{2+}. The bars represent incubation of MLB or washout; arrows represent the starts of stepwise applications of Ca^{2+} (0.125, 0.5, 1, 2, and 4 mM). (B) Effect of saline (○, as control), MLB (●) and verapamil (■) on vasoconstriction caused by Ca^{2+} in denuded artery rings ($n = 8$). $^{**}P < 0.01$, compared with control.

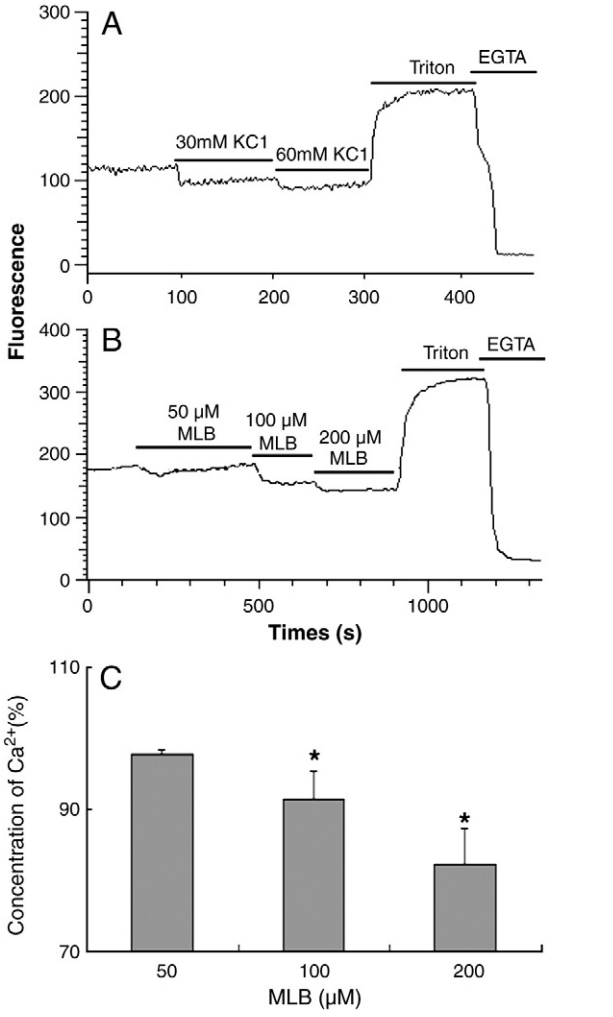

Fig. 3. MLB decreased intracellular Ca^{2+} concentration in endothelial cells. (A) Intracellular Ca^{2+} was decreased by high KCl. The bars represent applications of 30 and 60 mM KCl, triton and EGTA. (B) Intracellular Ca^{2+} was decreased by MLB. The bars represent applications of 50, 100, and 200 μM MLB, triton and EGTA. (C) MLB decreased intracellular Ca^{2+} in a concentration-dependent manner. Intracellular Ca^{2+} concentration before application of MLB was taken as 100% ($n = 7$). $^{*}P < 0.05$, compared with control.

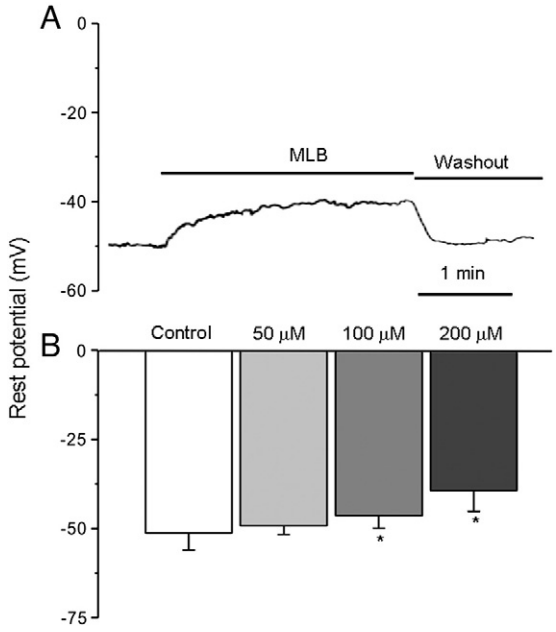

Fig. 4. The membrane potential of endothelial cells was depolarized by MLB. (A) The membrane potential changed following application of MLB. (B) MLB depolarized membrane potential in a concentration-dependent manner (MLB 50, 100, and 200 μM) ($n=8$). *$P<0.05$ compared with control.

2.9 mV ($n=11$). In current-clamp mode, the membrane potential of HMEC-1 cells was set to −30 mV, close to the resting membrane potential (Fig. 4).

3.4. MLB inhibited K^+ currents in HMEC-1 cells

A family of voltage-dependent outward currents was elicited by depolarizing the cell from a holding potential of −80 mV to a series of command potentials ranging from −80 to +50 mV. The outward currents were not inactivated during the 500 ms depolarization pulse and were sensitive to 10 mM TEA, which meant the primary component of the outward currents were K^+ currents. MLB produced a gradual voltage-dependent decrease of currents and the currents recovered upon washout (Fig. 5A). *I/V* relationship was expressed in terms of current density calculated using the membrane capacitance (Fig. 5B). A single depolarization pulse from −70 to +40 mV was adopted to obtain the concentration–response curves and the currents in control were taken as 100%. Results showed that the inhibitory effect of MLB on K^+ currents was concentration-dependent (Fig. 5C).

4. Discussion

In this study, MLB did not inhibit the stepwise contractions caused by Ca^{2+} influx in isolated mesentery arteries, in which case the smooth muscle cells were depolarized and Ca^{2+} flowed into cells via Ca^{2+} channels. This demonstrated that MLB did not block L-Ca^{2+} channels directly.

ECs are generally regarded as nonexcitable. No functional role exists for voltage-gated Ca^{2+} channels such as L-type and T-type Ca^{2+} channels in ECs (Adams et al., 1989; Takeda et al., 1987). In ECs, Ca^{2+} enters the cells via nonselective cation channels. In ECs loaded with Fluo-3, following the application of MLB, a significant stepwise decrease of fluorescence was recorded in a concentration-dependent manner, indicating that $[Ca^{2+}]i$ was reduced. This result was consistent with previous studies (Liu et al., 2007; Luo

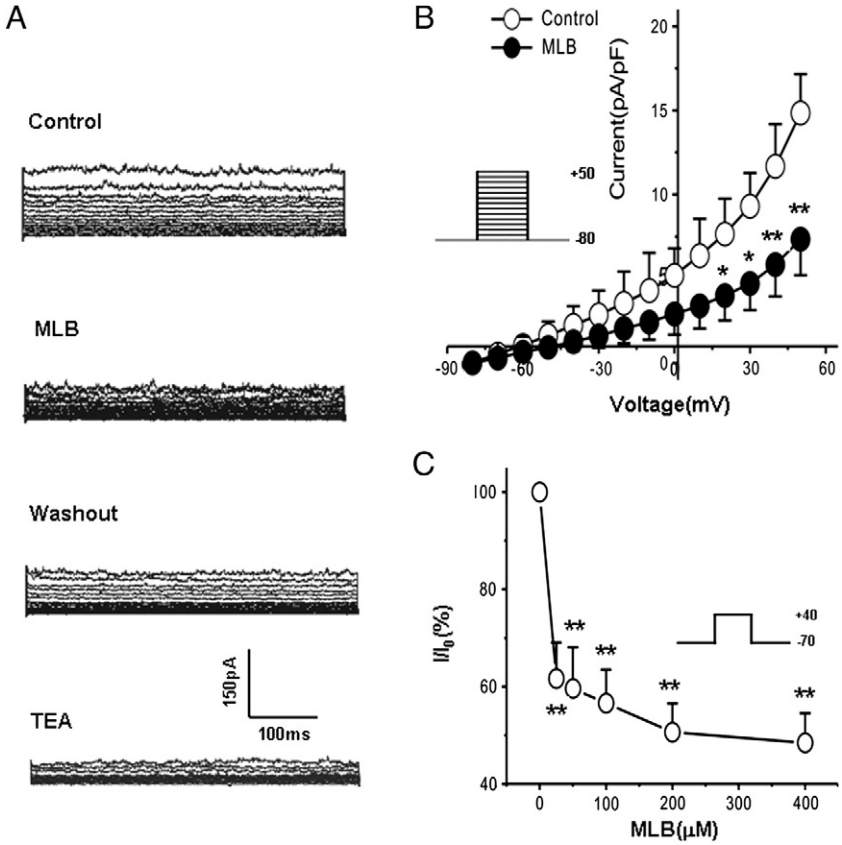

Fig. 5. MLB inhibited K^+ currents in endothelial cells. (A) The currents inhibited by MLB recovered upon washout and were sensitive to 10 mM TEA. (B) Current–voltage (*I/V*) curves. (C) Concentration–response curve (MLB 25, 50, 100, 200, and 400 μM) ($n=8$). *$P<0.05$, **$P<0.01$ compared with control.

and Wang, 2001) and showed that MLB could decrease $[Ca^{2+}]i$ in ECs in the absence of pathogenic factors.

Membrane potential, together with the transmembrane gradient of Ca^{2+}, provided the electrochemical driving force for extracellular Ca^{2+} to flow into ECs. Once membrane potential was depolarized, the electrochemical driving force for transmembrane passive inward Ca^{2+} leakage would decrease, and subsequently, $[Ca^{2+}]i$ would decrease (Laskey et al., 1990; Moore et al., 1998). In the patch-clamp experiment, it was demonstrated that the membrane potential of ECs was depolarized by MLB in a concentration-dependent manner. This result was consistent with results from $[Ca^{2+}]i$ measuring experiments. Combining the results of these two sets of experiments, we proposed that MLB depolarized membrane potential, decreased the driving force for Ca^{2+} and caused $[Ca^{2+}]i$ decrease.

Endothelial membrane potential was primarily controlled by K^+ channels. The outward whole-cell K^+ currents of ECs were inhibited by MLB in a concentration-dependent and voltage-dependent manner. K^+ currents recovered upon washout of MLB. Blocking K^+ channels induced depolarization of membrane potential. It is reasonable to presume that MLB inhibited K^+ currents, which caused depolarization of membrane potential and finally decreased $[Ca^{2+}]i$.

Membrane potential and $[Ca^{2+}]i$ were recorded from cells perfused with 30 mM K^+. In normal Krebs, MLB did not inhibit $[Ca^{2+}]i$, nor depolarized membrane potential (data not shown). This may be due to the state-dependent affinity of K^+ channel. Activation of K^+ channels may be necessary for MLB to bind to K^+ channels and block them.

ECs permeability and neutrophilic leukocyte diapedesis through paracellular gaps are cardinal features of acute inflammation. During inflammation, elevated $[Ca^{2+}]i$ decreased cAMP and increased intercellular permeability (Moore et al., 1998). The capability of MLB to decrease $[Ca^{2+}]i$ may provide it potential advantage to protect ECs from dysfunction during inflammation and arteriosclerosis. The significant anti-inflammation effects (Chen et al., 2006; Wang et al., 2007) of MLB was underpinned by its capability of decreasing $[Ca^{2+}]i$ found in our research.

It is generally agreed that synthesis and/or release of vasoactive agents depends on or can be modulated by changes in $[Ca^{2+}]i$ (Busse et al., 1991; Inagami et al., 1995; Nilius et al., 1997). Ca^{2+} influx triggers the production and release of numerous vasoactive agents from the endothelium including nitric oxide (Moore et al., 1998), vasoconstrictive prostaglandins, endothelins and tissue-type plasminogen activator. We propose here that MLB may play an important regular role in the activity of the cardiovascular system by affecting Ca^{2+} influx into ECs.

In conclusion, the present study suggests that MLB decreased $[Ca^{2+}]i$ by inhibiting K^+ currents and depolarizing membrane potential in ECs.

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