Effects of Magnesium Lithospermate B and its Analogues on Ca^{2+} Homeostasis in Cultured Rat Thoracic Aorta Vascular Smooth Muscle Cells

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Key words
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- Fluo-3

Abstract

Magnesium lithospermate B (MLB), sodium rosmarinate (SR), and magnesium lithospermate (ML) are biologically active components isolated from aqueous extracts of the Chinese medicine Danshen, the dried root and rhizome of *Salvia miltiorrhiza* Bunge (Labiatae). These compounds share similar chemical structures of polyphenols and oligomer condensates with caffeic acid. In this study, we compared the effects of MLB, ML, and SR on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in cultured rat thoracic aorta vascular smooth muscle cells (VSMCs), using the Ca^{2+}-sensitive dye, Fluo-3, as an indicator. In our experiments, MLB, ML, and SR did not affect the basal value of $[Ca^{2+}]_i$ in VSMCs. In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ increase in VSMCs induced by 20 µM ATP was attenuated by MLB, ML, and SR in a dose-dependent manner. Moreover, under this condition, MLB suppressed the increase in $[Ca^{2+}]_i$ in VSMCs induced by 1 µM thapsigargin, but not ML or SR. In the presence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ increase in VSMCs induced by 60 mM KCl was attenuated by MLB and SR in a dose-dependent manner, but not by ML. These results suggest that MLB, ML and SR regulate Ca^{2+} homeostasis in VSMCs via different pathways. Our findings should aid in elucidating the mechanisms of the vasodilator action of aqueous extracts of *Salvia miltiorrhiza*.

Introduction

Danshen, the dried root and rhizome of the plant *Salvia miltiorrhiza* (Labiatae) [1], is a traditional Chinese herbal medicine used for the treatment of cardiovascular diseases. The pharmacological activities of MLB (**Fig. 1**), a major component of the aqueous extract of *Salvia miltiorrhiza* [2], have been extensively investigated. MLB (also named salvianolic acid B) possesses several medicinal properties, such as hepatoprotection [3], vasorelaxation [4], antihypertension [5], and free radical scavenging activities [6, 7]. Changes in $[Ca^{2+}]_i$ constitute the principal mechanisms regulating the contractile state of VSMCs [8]. We speculate that MLB exerts a vasorelaxation effect by preventing Ca^{2+} overload in vascular smooth muscle cells. Moreover, MLB behaved in a Ca^{2+} antagonistic fashion in a voltage-dependent manner in ventricular myocytes [9] and inhibited extracellular calcium influx in endothelial cells and platelets [10–12]. In addition, salvianolic acid B prevented 6-OHDA-induced $[Ca^{2+}]_i$ elevation in SH-SY5Y cells [13] and inhibited H_2O_2- and $A\beta_{25-35}$-induced $[Ca^{2+}]_i$ elevation in PC12 cells [14, 15]. Accordingly, we examined the hypothesis that MLB modulates Ca^{2+} homeostasis in VSMCs, similar to ventricular myocytes, endothelial cells, platelets, SH-SY5Y cells and PC12 cells.

ML and SR are two additional biologically active components of the aqueous extract of *Salvia miltiorrhiza* [1]. ML has antioxidative and anti-inflammatory activities [16], as well as hormone regulatory effects [17]. In recent years, ML has attracted considerable interest due to its anti-HIV activity [18]. However, to our knowledge, no studies on the effects of ML on Ca^{2+} regulation in VSMCs have been reported to date. SR possesses antithrombotic [19], antiallergic [20], and antiviral properties [21]. In addition, SR inhibited T-cell antigen receptor-induced $[Ca^{2+}]_i$ increases in Jurkat T cells [22–24]. MLB, ML, and SR share similar chemical structures of polyphenols and oligomer condensates with caffeic acid (**Fig. 1**). It would be of interest to determine whether these compounds have comparable effects on $[Ca^{2+}]_i$ as MLB, in view of the similarities in their chemical structures. In this study, we compared the effects

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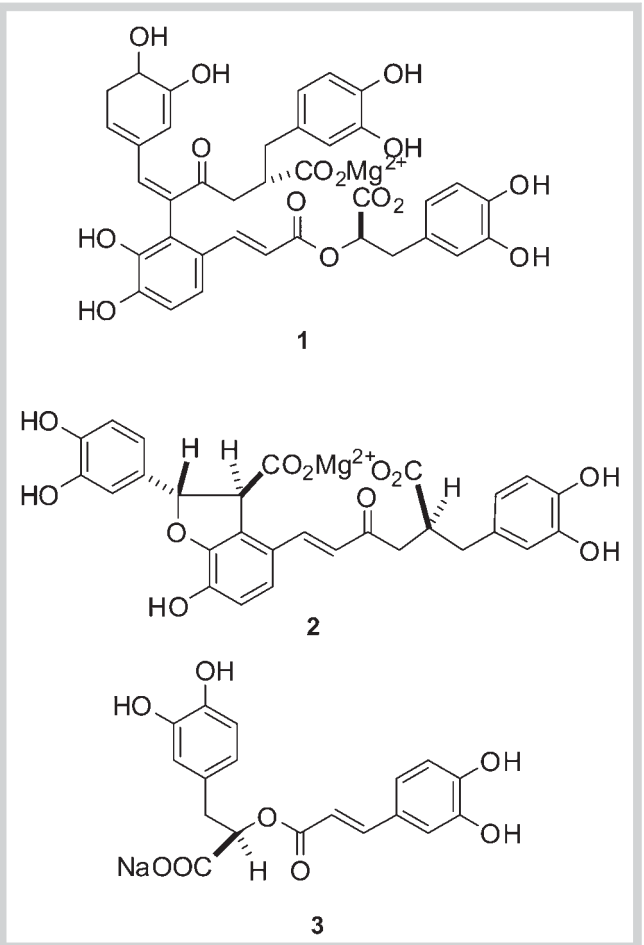

Fig. 1 Chemical structures of magnesium lithospermate B (**1**), magnesium lithospermate (**2**), and sodium rosmarinate (**3**).

of MLB, ML, and SR on intracellular calcium mobilization in VSMCs.

Materials and Methods

Plant material

The roots of *Salvia miltiorrhiza* were collected in Shandong, China, in November 2006, and identified by Prof. Heming Yang. A voucher specimen (No. SIMMSM06) is deposited at the Herbarium of Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China.

Extraction and isolation

Air-dried roots (5 kg) were pulverized and extracted with 80% aqueous acetone at room temperature for 3 × 24 h. After concentration under vacuum to remove the organic solvent, the suspended residue was removed by centrifugation. The aqueous solution was subjected to MCI gel CHP-20P column chromatography (10 cm i.d. × 50 cm) using MeOH/H_2O (0% to 100% gradient) as eluent to give seven fractions (A – G). Fraction C (6.8 g) was chromatographed on a Chromatorex C18-OPN column (5 cm i.d. × 30 cm) with H_2O and 10, 20, 30 and 40% MeOH (each 400 mL) as eluent to give three subfractions (I – III). Fraction III was further separated over a TSK gel Toyopearl HW-40F column (2.0 cm i.d. × 25 cm), eluted with MeOH-H_2O (1 : 9, 1 : 4, 3 : 7, each 300 mL), and Sephadex LH-20 column (1.5 cm i.d. × 70 cm) eluted with 95% EtOH to yield compound ML (2.0 g). Fraction D (6.5 g) was subjected to passage over a Chromatorex C18-OPN column (3 cm i.d. × 25 cm) chromatography eluted with MeOH-H_2O (1 : 9, 1 : 4, 3 : 7, 2 : 3, 1 : 1, each 300 mL) to give four fractions (I – IV). Fraction IV (900 mg) was further subjected to TSK gel Toyopearl HW-40F (2.0 cm i.d. × 25 cm) eluted with MeOH-H_2O (5%, 10%, 15%, 20%, each 250 mL), and further purified by Sephadex LH-20 column (1.5 cm i.d. × 60 cm) chromatography with 95% EtOH as eluent to afford compound MLB (65.8 mg). Compound SR (203 mg) was obtained from fraction F (3.4 g) by Chromatorex C18-OPN column chromatography (2 cm i.d. × 30 cm) using MeOH-H_2O (3 : 7, 2 : 3, 1 : 1, 3 : 2, 7 : 3, each 300 mL) as solvents. Compounds MLB, ML, and SR displayed > 99.4% purity as confirmed with HPLC analyses, and their structures were identified by spectral analyses and compared with those of their acid forms in the literature [25,26], and the metal ion was determined by atomic absorbance spectrum.

Preparation of free acids of MLB, ML, and SR: 10 mg MLB, ML, or SR were mixed with 2 mL H^+-type cation ion exchange resin (Dowex 50 WX4) and 10 mL acetone. After being stirred for 30 min at room temprature, the clarified acetone solution was filtered and evaporated to afford lithospermic acid B, lithosperic acid, and rosmarinic acid.

Animals

Male Sprague-Dawley (SD) rats (Shanghai SLAC Laboratory Animal Co.) weighing 150–180 g were housed in cages, maintained at 25 °C with 12 h of light, and were allowed free access to water and standard rat chow. All experimental procedures conformed with the institutional guidelines on the care and use of experimental animals set by the Chinese Academy of Sciences.

Cell culture and reagents

Primary vascular smooth muscle cells were obtained from the thoracic aorta of SD rats via the tissue explants method, as described previously [27]. More than 98% of the cells were positive for smooth muscle-specific α-actin, and exhibited the typical hill-and-valley morphology of VSMCs. Cells at passages 3 to 5 were used for experiments. Triton X-100, EGTA, ATP, thapsigargin, and verapamil (purity ≥ 99.0%) were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) was acquired from Sijiqing Biotech Co., Ltd. Fluo-3/AM was the product of Biotium. DMEM was purchased from Gibco, while monoclonal mouse anti-α-smooth muscle actin was from Wuhan Boster Biological Technology Co., Ltd.

Measurement of $[Ca^{2+}]_i$ in VSMCs with Fluo-3

$[Ca^{2+}]_i$ in VSMCs was measured using the calcium-sensitive dye, Fluo-3-acetoxymethyl ester (Fluo-3/AM), as described previously [28]. Cells were incubated in DMEM with 2 µmol/L membrane-permeable Fluo-3/AM in the dark at 37 °C for 40 min. Cells were subsequently washed twice gently with Ca^{2+}-free or normal Krebs solution after centrifugation at 2000 rpm for 10 min, and maintained in the same solution. The Krebs solution contained (in mM): 1.8 $CaCl_2$, 112 NaCl, 5 KCl, 1 $MgCl_2{\cdot}6\,H_2O$, 5 HEPES, 15 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 $NaH_2PO_4{\cdot}2\,H_2O$ and 11 glucose, pH 7.4. Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and adding 0.5 mM EGTA. Low Na^+ solution was made by an equimolar substitution of LiCl for NaCl. Cells were incubated on ice until the measurement of $[Ca^{2+}]_i$.

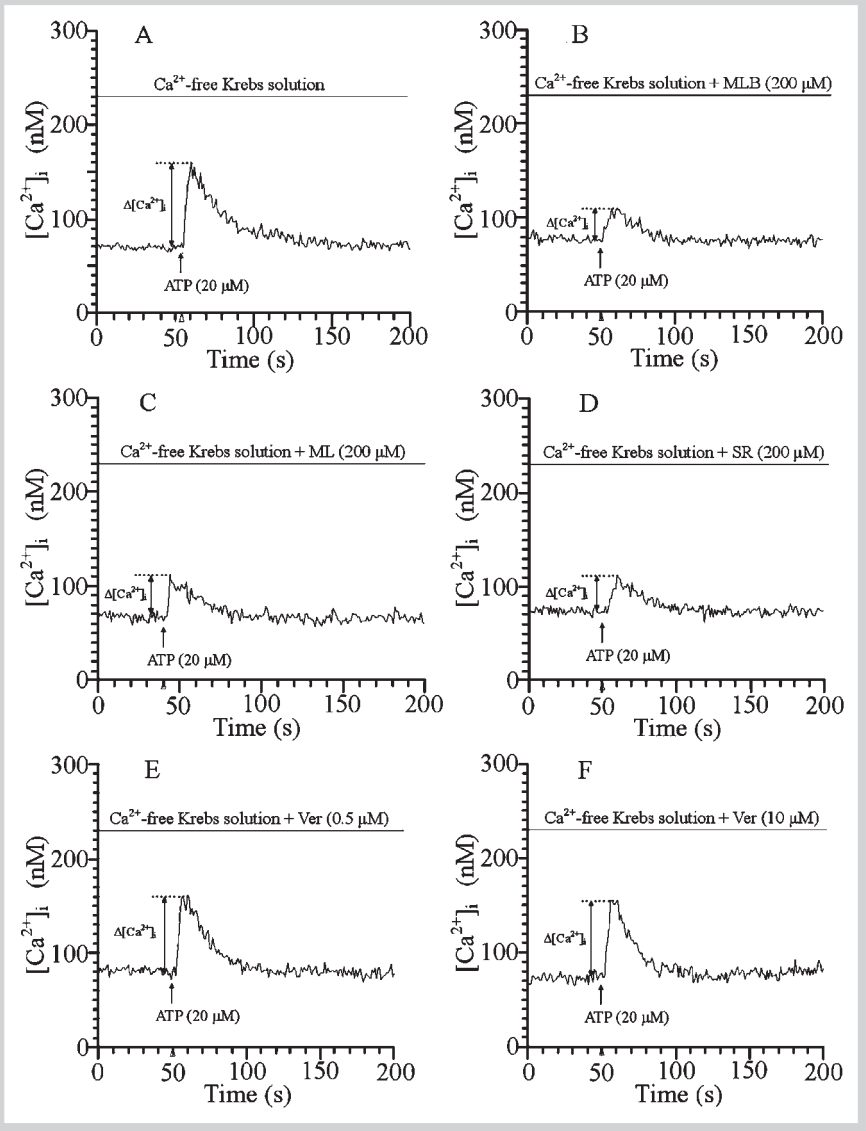

Fig. 2 Effects of MLB, ML, SR on ATP-induced $[Ca^{2+}]_i$ increase in cells incubated with Ca^{2+}-free Krebs solution. **A** Fluo-3-loaded cells were suspended in Ca^{2+}-free Krebs solution. After thermal equilibration at 37 °C, ATP (final concentration, 20 µM) was added to the rapidly stirred cell suspension at the indicated time. **B, C** and **D** Cells were pretreated with 200 µM MLB, ML or SR for 5 min at 37 °C, prior to the experiment. **E, F** Cells were pretreated with 0.5 µM or 10 µM verapamil for 5 min at 37 °C, prior to the experiment.

$[Ca^{2+}]_i$ was measured at an excitation wavelength of 506 nm and emission wavelength of 527 nm in a Hitachi F-4500 spectrophotometer. The temperature of the cell suspensions during measurement was 37 °C. Maximal fluorescence (F_{max}) was estimated by adding 0.2 % Triton X-100, and minimal fluorescence (F_{min}) by adding 5 mM EGTA. Fluorescence-time curves were converted to $[Ca^{2+}]_i$-time curves using the Intracellular Cation Measurement Program, and $[Ca^{2+}]_i$ was calculated according to the formula:

$$[Ca^{2+}]_i = K_d \times (F - F_{min})/(F_{max} - F)$$

where K_d represents the dissociation constant of Fluo-3 for Ca^{2+}, assumed as 385 nM.

Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed using ANOVA. The intergroup comparisons (post hoc analysis) among the data with equal variances were made by the LSD method, while Tamhane's T2 method was used for the data with unequal variances. A value of $p < 0.05$ was considered significant.

Results and Discussion

In the initial stages of our study, the cytotoxicities of MLB, ML and SR against VSMCs were assessed after 24 h of incubation using the MTT assay. Cell viability remained unaffected in the presence of up to 200 µM MLB, ML and SR (data not shown). Consequently, concentrations of 50–200 µM were employed, ruling out the possible contribution of cytotoxicity in attenuating the $[Ca^{2+}]_i$ increase induced by agonists.

In control experiments, application of ATP at a final concentration of 20 µM induced a steep increase in $[Ca^{2+}]_i$ from a basal level of 72.8 ± 8.9 nM to 154.0 ± 12.0 nM (**Fig. 2A**). The peak amplitude of the net $[Ca^{2+}]_i$ increase ($\Delta[Ca^{2+}]_i$) was 81.2 ± 7.5 nM (**Table 1**). Following exposure of cells to MLB, ML or SR for 5 min, 20 µM ATP induced similar shapes of Ca^{2+} transients (**Fig. 2**). However, the amplitudes of MLB-, ML- and SR-treated cells were smaller than those in control cells (**Table 1**). Since MLB, ML and SR are polyphenols and oligomer condensates of caffeic acid, we speculate that the inhibitory effects on ATP-induced calcium increase are attributable to the similar chemical structures.

Thapsigargin, a selective inhibitor of sarcoplasmic Ca^{2+}-ATPase, induced Ca^{2+} transients in control cells incubated with Ca^{2+}-free

Table 1 Effects of the indicated concentrations of drugs on $\Delta[Ca^{2+}]_i$ evoked by ATP, thapsigargin and KCl in VSMCs.

	Changes in intracellular calcium concentration ($\Delta[Ca^{2+}]_i$ nM)		
	ATP (20 μM)	thapsigargin (1 μM)	KCl (60 mM)
Control	81.2 ± 7.5	75.4 ± 10.4	78.8 ± 8.5
MLB (μM)			
50	60.1 ± 5.4**	64.4 ± 6.0	65.0 ± 8.7
100	44.0 ± 5.3**	53.6 ± 2.1 **	51.7 ± 8.3 **
200	37.0 ± 5.5**	48.6 ± 4.9 **	42.5 ± 9.1 **
ML (μM)			
50	68.2 ± 7.9	69.3 ± 10.4	81.2 ± 10.6
100	64.2 ± 8.9 *	69.1 ± 8.8	80.4 ± 13.1
200	38.0 ± 4.8 **	65.9 ± 10.4	73.6 ± 12.9
SR (μM)			
50	56.0 ± 10.6 **	68.2 ± 10.9	61.0 ± 13.4
100	39.2 ± 9.0 **	66.1 ± 11.5	48.8 ± 7.2**
200	35.1 ± 7.0 **	66.2 ± 9.8	39.6 ± 11.2 **
Ver (μM)			
0.5	77.7 ± 9.5	71.3 ± 7.5	36.3 ± 3.5 **
10	72.9 ± 7.8	71.7 ± 6.8	3.5 ± 2.5 **

Data of mean with S. D. were obtained from six separate experiments. * $P < 0.05$ and ** $p < 0.01$ compared with control in each group, respectively

Table 2 Effects of the indicated concentrations of drugs on resting state $[Ca^{2+}]_i$ in VSMCs.

	Intracellular calcium concentration ($[Ca^{2+}]_i$ nM)	
	in Ca^{2+}-free Krebs solution	in normal-Ca^{2+} Krebs solution
Control	71.8 ± 8.5	143.6 ± 13.5
MLB (μM)		
50	76.3 ± 7.0	143.6 ± 10.8
100	72.9 ± 8.1	145.5 ± 9.6
200	73.1 ± 4.7	141.6 ± 7.8
ML (μM)		
50	71.6 ± 6.6	134.5 ± 7.9
100	72.9 ± 4.4	137.4 ± 8.3
200	73.2 ± 5.0	138.2 ± 8.9
SR (μM)		
50	77.0 ± 6.1	157.4 ± 7.8
100	75.7 ± 10.3	157.9 ± 10.5
200	77.3 ± 9.1	149.6 ± 16.2
Ver (μM)		
0.5	74.5 ± 8.3	139.7 ± 15.6
10	70.3 ± 10.8	134.1 ± 17.6

Data of mean with S. D. were obtained from six separate experiments. * $P < 0.05$ and ** $p < 0.01$ compared with control in each group, respectively

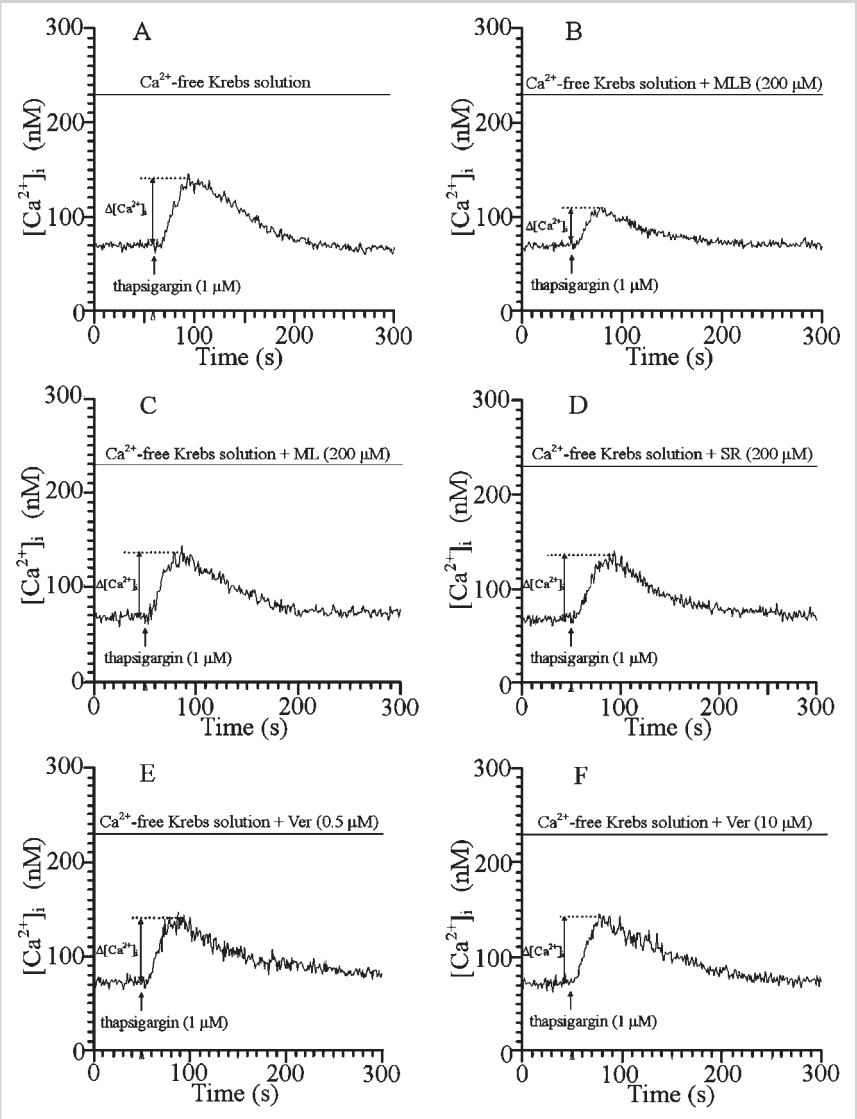

Fig. 3 Effects of MLB, ML, SR on thapsigargin-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free Krebs solution. **A** Fluo-3 loaded cells were suspended in Ca^{2+}-free Krebs solution. After thermal equilibration at 37 °C, thapsigargin (final concentration, 1 μM) was added to the rapidly stirred cell suspension at the indicated time. **B, C** and **D** Cells were pretreated with 200 μM MLB, ML or SR for 5 min at 37 °C, prior to the experiment. **E, F** Cells were pretreated with 0.5 μM or 10 μM verapamil for 5 min at 37 °C, prior to the experiment.

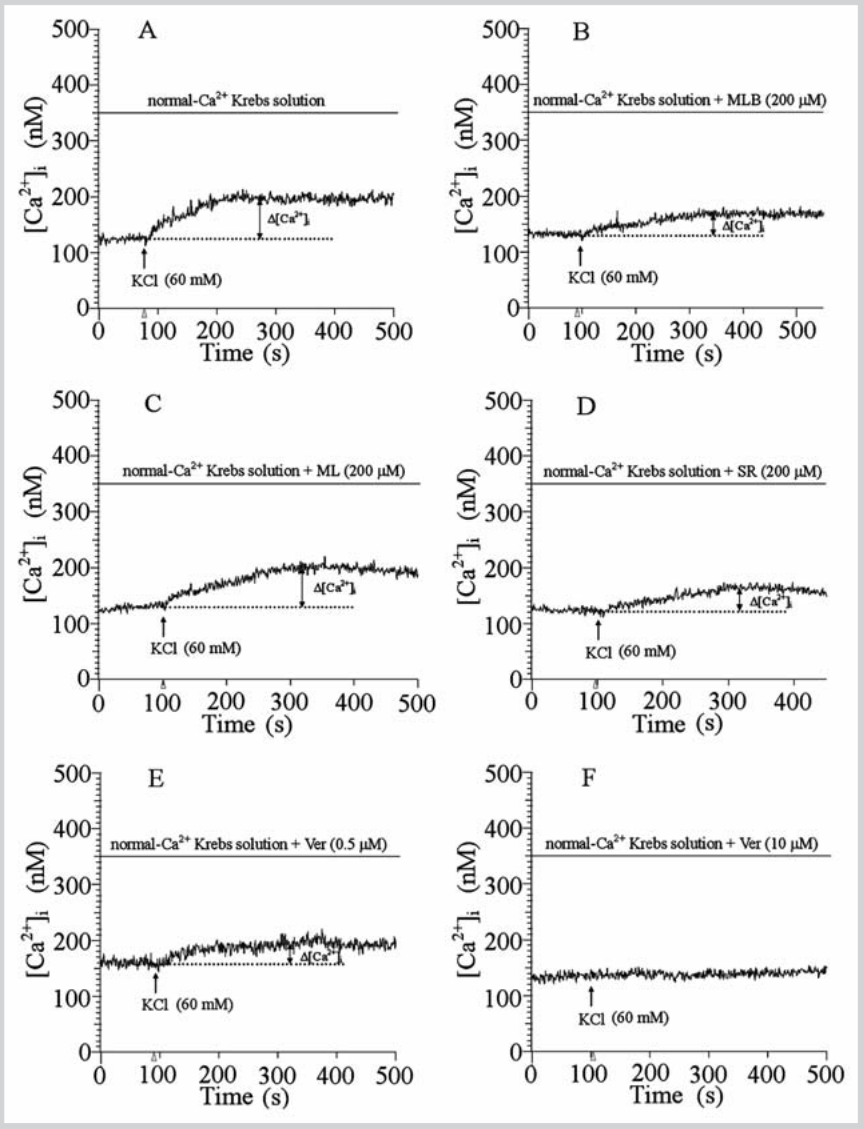

Fig. 4 Effects of MLB, ML, SR on KCl-induced $[Ca^{2+}]_i$ increase in cells treated with normal-Ca^{2+} Krebs solution. **A** Fluo-3 loaded cells were suspended in normal Ca^{2+} Krebs solution. After thermal equilibration at 37 °C, KCl (final concentration, 60 mM) was added to the rapidly stirred cell suspension at the indicated time. **B, C** and **D** Cells were pretreated with 200 μM MLB, ML or SR for 5 min at 37 °C, prior to the experiment. **E, F** Cells were pretreated with 0.5 μM or 10 μM verapamil for 5 min at 37 °C, prior to the experiment.

Krebs solution (**Fig. 3 A**). The peak amplitude of the net $[Ca^{2+}]_i$ increase ($\Delta[Ca^{2+}]_i$) was 75.4 ± 10.4 nM (**Table 1**). MLB attenuated calcium transients induced by thapsigargin (1 μM) in a dose-dependent manner, but not ML or SR (**Fig. 3**, **Table 1**). MLB possesses the chemical structure of polyphenols, and is a large molecule with strong polarity. Consequently, it would be difficult for MLB to enter cells and directly affect the leakage of calcium from sarcoplasmic calcium stores induced by thapsigargin. Plasma membrane Ca^{2+}-ATPase (PMCA) pump, SR Ca^{2+}-ATPase (SERCA) pump, Na^+/Ca^{2+} exchanger (NCX), and cytosolic Ca^{2+}-binding proteins are involved in reducing the cytosolic calcium level [29]. We speculate that these mechanisms are involved in mediating the ability of MLB to suppress thapsigargin-induced $[Ca^{2+}]_i$ increase in VSMCs. Accordingly, we examined this hypothesis by inhibiting NCX with low Na^+ solution and PMCA with vanadate. In our study, 1 mM vanadate attenuated the inhibitory effect of MLB on thapsigargin-induced $[Ca^{2+}]_i$ increase (**Fig. 5 B** and **Table 3**) while low Na^+ solution did not (**Fig. 5 C** and **Table 3**). Our results indicate that PMCA is involved in the reduction of the thapsigargin-induced Ca^{2+} increase.

Influx of extracellular Ca^{2+} plays a pivotal role in vascular smooth muscle contraction. The aqueous extract of Danshen and salvianolic acid B relax rat coronary and rat femoral arteries via inhibition of Ca^{2+} influx in VSMCs [4,30]. Previous studies by our group showed that MLB modulates L-type calcium current in guinea pig ventricular myocytes [9]. High K^+ solution was applied to activate voltage-dependent Ca^{2+} channels and induced Ca^{2+} influx. $[Ca^{2+}]_i$ was gradually increased in response to 60 mM KCl in

Table 3 Changes in $[Ca^{2+}]_i$ response to MLB in VSMCs treated with vanadate or low $[Na^+]_o$ in combination.

	Thapsigargin induced changes in intracellular calcium concentration ($\Delta[Ca^{2+}]_i$ nM)		
	Vehicle	Vanadate	Low $[Na^+]_o$
Control	75.8 ± 7.9	84.9 ± 9.7	83.7 ± 9.5
MLB (μM)			
100	53.5 ± 7.9**	72.8 ± 5.7	60.5 ± 6.7 **
200	46.4 ± 4.3 **	71.8 ± 7.9	54.6 ± 6.6 **

Data of mean with S.D. were obtained from six separate experiments. * $P < 0.05$ and ** $p < 0.01$ compared with control in each group, respectively. Vehicle group cells were neither treated with vanadate nor low Na^+ solution before stimulation with thapsigargin

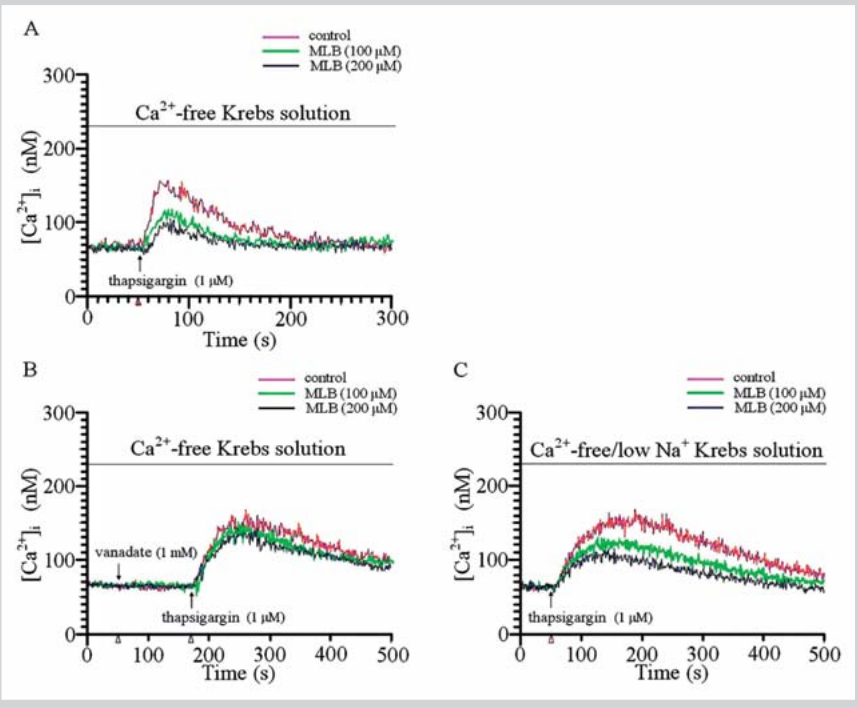

Fig. 5 Effects of vanadate (PMCA blocker) or low extracellular Na^+ concentration ($[Na^+]_o$) on MLB-induced $[Ca^{2+}]_i$ decrease in VSMCs. **A** Fluo-3 loaded cells were suspended in Ca^{2+}-free Krebs solution. After pretreatment with or without MLB for 5 min, thapsigargin (1 μM) was added to the rapidly stirred cell suspension at the indicated time. **B** Fluo-3 loaded cells were suspended in Ca^{2+}-free Krebs solution. After pretreatment with or without MLB for 5 min, vanadate (1 mM) and thapsigargin (1 μM) were added at the indicated time respectively. **C** Fluo-3 loaded cells were suspended in Ca^{2+}-free/low Na^+ Krebs solution. After pretreatment with or without MLB for 5 min, thapsigargin (1 μM) was added at the indicated time.

control cells from a basal level of 143.6 ± 13.5 nM to 222.3 ± 16.3 nM (**Fig. 4A**). MLB and SR attenuated elevation of $[Ca^{2+}]_i$ induced by 60 mM KCl in a concentration-dependent manner (**Table 1**). However, ML did not have an effect, even at the highest concentration (200 μM) (**Fig. 4C** and **Table 1**). This finding supports the theory that inhibition of L-type voltage-operated Ca^{2+} channels is an important mechanism underlying the vasodilator activity of salvianolic acid B [4].

After treatment with the indicated concentrations of MLB, ML or SR at 37 °C for 5 min, no differences in resting $[Ca^{2+}]_i$ of cells incubated with Ca^{2+}-free Krebs solution were observed among the groups. Basal $[Ca^{2+}]_i$ was 71.8 ± 8.5 nM in control cells. $[Ca^{2+}]_i$ in 200 μM MLB, ML or SR-treated cells remained at 73.1 ± 4.7 nM, 73.2 ± 5.0 nM, and 77.3 ± 9.1 nM, respectively (**Table 2**). In the presence of normal Ca^{2+} Krebs solution, basal $[Ca^{2+}]_i$ was 143.6 ± 13.5 nM in control cells. We observed no significant differences in resting $[Ca^{2+}]_i$ between control and MLB, ML or SR-treated cells in normal-Ca^{2+} Krebs solution (**Table 2**). This finding implies that none of these compounds affect the spontaneous influx of extracellular Ca^{2+} and release of Ca^{2+} from internal stores.

We used L-type calcium channel antagonist verapamil as a reference compound in this study. Although the inhibition effects of MLB and RA on KCl-induced calcium influx are weak as compared with that of verapamil, MLB and its analogues can inhibit calcium release from intracellular stores while verapamil cannot (**Table 1**).

The serum concentration of MLB in beagle dogs after a bolus injection of 3–12 mg/kg MLB was reported as 24 to 107 mg/L (approximately 32–144 mM) [31]. MLB at concentrations of 50 to 200 μM effectively inhibited both calcium release and calcium influx. It is conceivable that MLB inhibits $[Ca^{2+}]_i$ increase at therapeutic concentrations, resulting in vasodilatory activity.

In conclusion, our findings confirm that three biologically active components of the aqueous extract of *Salvia miltiorrhiza*, MLB, ML and SR, regulate Ca^{2+} homeostasis in rat vascular smooth muscle cells. In terms of calcium modulation activity, MLB is more effective than ML and SR. Moreover, the effects of MLB, ML and SR on $[Ca^{2+}]_i$ in VSMCs are multifactorial. The exact mechanisms by which MLB, ML or SR regulate $[Ca^{2+}]_i$ remain to be established. Additionally, while the hypotension and vasodilator actions of the water-soluble fraction of Danshen and MLB have been extensively analyzed, the effects of ML and SR on vascular diseases are yet to be established. Our preliminary results indicate that ML and SR have beneficial effects, and are thus applicable in antihypertensive treatment. Furthermore, the present findings are useful for clarifying the mechanisms underlying the vasodilatory action of the aqueous extract of *Salvia miltiorrhiza*.

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