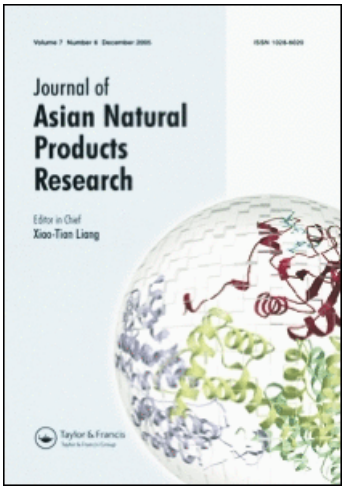

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Bang-Hao Zhu[a]; Li Ma[a]; Xue-Diao Pan[a]; Yu-Ling Huang[a]; Jie Liu[a]
[a] Department of Pharmacology, Sun Yat-Sen Medical School, Sun Yat-Sen University, Guangzhou, China

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Bang-Hao Zhu*, Li Ma, Xue-Diao Pan, Yu-Ling Huang and Jie Liu

Department of Pharmacology, Sun Yat-Sen Medical School, Sun Yat-Sen University, Guangzhou 510080, China

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This study was designed to investigate the effect of scutellarin (**1**) on the modulation of intracellular Ca^{2+} concentration in thoracic smooth muscle cells of rat. Single smooth muscle cells were obtained enzymatically. Fluo-3 AM was used to determine the alteration of intracellular-free Ca^{2+} ($[Ca^{2+}]_i$) and the changes in fluorescence intensity under different agonists were recorded. Compound **1** induced Ca^{2+} transients in the medium with and/or without Ca^{2+}. In the Ca^{2+}-free medium, after pretreatment of **1**, thapsigargin failed to cause the elevation of $[Ca^{2+}]_i$. However, **1** still caused the elevation of $[Ca^{2+}]_i$ after pretreatment of thapsigargin. The infusion of **1** blocked KCl-induced Ca^{2+} entry and this effect was hardly reversible. The results of present study suggested that **1** increased $[Ca^{2+}]_i$ by blocking sarcoplasmic reticulum Ca^{2+}/ATPase and blocked voltage-dependent Ca^{2+} channels in smooth muscle cells of the rat thoracic aortic artery.

Keywords: scutellarin; intracellular-free Ca^{2+}; KCl; thapsigargin; vascular smooth muscle cell

1. Introduction

Scutellarin (**1**) is one of the flavonoids isolated from the Chinese herb *Erigeron breviscapus*, which has long been used clinically to treat cardiovascular diseases, cerebral infarction and stroke in China. However, its therapeutic mechanism is still not well known. Studies have suggested that **1** can increase cerebral blood flow, improve microcirculation, inhibit protein kinase C and prevent ischemic cerebral cell apoptosis.[1–3] Moreover, it has been reported that **1** can protect cortical neurons from injury via decreasing intracellular Ca^{2+} overloading induced[4] by H_2O_2. Previously, we found that **1** can prevent the endothelial dysfunction in diabetic rats,[5] which suggested a potential role of **1** in treating diabetic complications.

Ca^{2+} is a ubiquitous second messenger controlling a broad range of cellular functions. In smooth muscle, contractions of the smooth muscle cells are regulated by the rise and fall of $[Ca^{2+}]_i$. The Ca^{2+} release from the sarcoplasmic reticulum stores and the influx from the extracellular stores contribute to the rise of $[Ca^{2+}]_i$. However, the Ca^{2+} uptake performed by Ca^{2+} ATPase, which pumps Ca^{2+} back into sarcoplasmic reticulum Ca^{2+} stores, contributes to the fall of $[Ca^{2+}]_i$. The sarcoplasmic reticulum Ca^{2+}/ATPase inhibitor, like thapsigargin, can cause the depletion of thapsigargin-sensitive sarcoplasmic reticulum Ca^{2+} stores and the rise of $[Ca^{2+}]_i$. Examining the effect of **1** on intracellular Ca^{2+} regulation would be essential for understanding its therapeutic mechanism.

*Corresponding author. Email: zhubh@mail.sysu.edu.cn

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The present study was undertaken in vascular smooth muscle cells (VSMC) isolated from rat thoracic aortic artery.

2. Results and discussion

2.1 Ca^{2+} transients induced by 1

Since the rise of $[Ca^{2+}]_i$ occurs as a result of both Ca^{2+} entry from the extracellular domains and Ca^{2+} release from intracellular stores, the first experiment was carried out to measure $[Ca^{2+}]_i$ during various concentrations of **1** treatment, in Ca^{2+}-containing buffer. As shown in Figure 1(A), **1** (0.1–100 μM) evoked a sequence of transient increases in $[Ca^{2+}]_i$. The same experiment was also carried out in Ca^{2+}-free buffer, as shown in Figure 1(B). There were no significant differences observed between these two responses (Figure 1(C)).

2.2 The interaction between 1 and thapsigargin-sensitive Ca^{2+} ATPase

Based on the results mentioned above, we concluded that the extracellular Ca^{2+} has nothing to do with the **1**-induced $[Ca^{2+}]_i$ elevation. Subsequently, we investigated the mechanism of **1**-induced $[Ca^{2+}]_i$ elevation in Ca^{2+}-free buffer and found an inhibition of **1** on Ca^{2+} ATPase. As shown in Figure 2, we investigated the interaction between **1** and thapsigargin-sensitive Ca^{2+} ATPase. In the Ca^{2+}-free perfusion solution, 50 μM of **1** was added twice to deplete the intracellular Ca^{2+}-stores. Under this condition, there was almost no response to thapsigargin (Figure 2(A),(B)), which suggested that **1** depleted thapsigargin-sensitive Ca^{2+} stores. Conversely, thapsigargin was added thrice to assure that the thapsigargin-sensitive Ca^{2+} stores were depleted, and then 50 μM of **1** could still

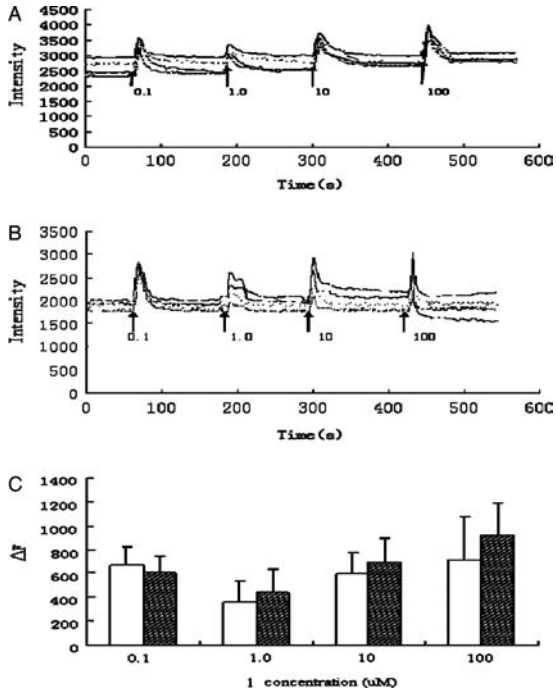

Figure 1. Elevations of fluorescence ratio induced by various concentrations of **1** in Ca^{2+}-containing or Ca^{2+}-free buffer. (A) Representative traces of $[Ca^{2+}]_i$ of four cells treated with 0.1–100 μM of **1** in Ca^{2+}-containing buffer. (B) Representative traces of $[Ca^{2+}]_i$ of four cells treated with 0.1–100 μM of **1** in Ca^{2+}-free buffer. (C) Elevation of fluorescence ratio induced by 0.1–100 μM of **1** in Ca^{2+}-containing (blank column) and Ca^{2+}-free (scratched column) buffer. Each column represents data from at least four different experiments and 20 cells.

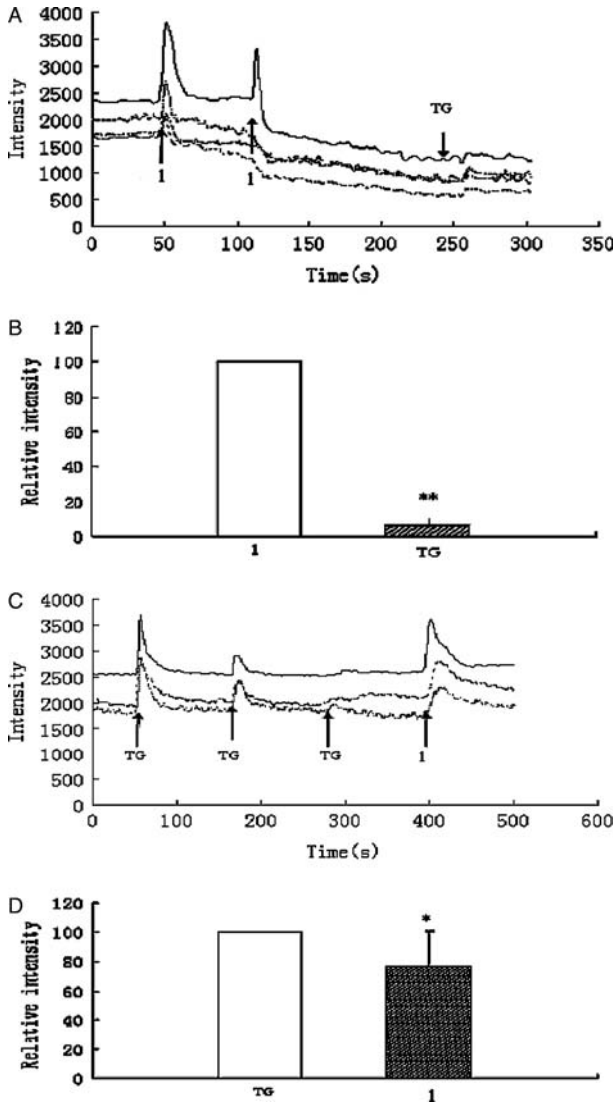

Figure 2. (A) Representative traces of $[Ca^{2+}]_i$ of four cells treated with 50 μM of **1** twice and successively with 5 μM TG (thapsigargin). (B) Elevation of fluorescence ratio during treatment with 5 μM TG after pretreatment with **1**, the values of TG represent the percentages by that of first **1** responses, data come from at least four different experiments and 20 cells. (C) Representative traces of $[Ca^{2+}]_i$ of three cells treated with TG at a final concentration of 5 μM and successively with 100 μM of **1**. (D) Elevation of fluorescence ratio during treatment with 100 μM of **1** after pretreatment with TG, the values of **1** represent the percentages by that of first TG responses, data come from at least four different experiments and 20 cells. $^{*}P < 0.05$ compared with TG-treated group, $^{**}P < 0.01$ compared with the **1**-treated group.

cause the elevation of $[Ca^{2+}]_i$ (Figure 2(C),(D)). The effect of **1** followed by thapsigargin treatment shows that **1** has additional, thapsigargin-insensitive targets.

2.3 Effect of 1 on KCl-induced Ca^{2+} influx

A high concentration of KCl can cause the depolarization of cell membrane, thereby

opening the L-type voltage-dependent Ca^{2+} channel. In the present study, when the cells were exposed to a solution containing 60 mM KCl twice, a repeatable elevation of $[Ca^{2+}]_i$ was observed (Figure 3(A)). However, the treatment of **1** during the first KCl infusion blocked the elevation of $[Ca^{2+}]_i$, and the second infusion of KCl hardly caused the elevation of $[Ca^{2+}]_i$ (Figure 3(B),(C)). These results indicate that **1** irreversibly blocked the depolarization-induced Ca^{2+} entry.

The major findings of the present study are as follows. First, the elevation of $[Ca^{2+}]_i$ is caused by **1**. To make sure that the rise in $[Ca^{2+}]_i$ caused by **1** was not the cause of the mechanical disturbance of the cells, we expose cells to solutions containing zero concentration of **1** and found no rise in $[Ca^{2+}]_i$ at all. Comparing the $[Ca^{2+}]_i$ responses with **1** obtained in Ca^{2+}-free and Ca^{2+}-containing buffer (Figure 1(C)), we found that there was no contribution of extracellular Ca^{2+} to the **1**-induced elevation of $[Ca^{2+}]_i$. Therefore, these data demonstrated the ability of **1** to release Ca^{2+} from intracellular stores. Second, the effects of **1** partially overlap with those of thapsigargin. Thapsigargin is believed to selectively block Ca^{2+} ATPase of the sarcoplasmic reticulum.[11] Compound **1** abolished the effects of following treatment with thapsigargin, but can still increase $[Ca^{2+}]_i$ after pretreatment with thapsigargin, which suggested that **1** may have additional targets besides the thapsigargin-sensitive Ca^{2+} ATPase. Using the same protocols, our results

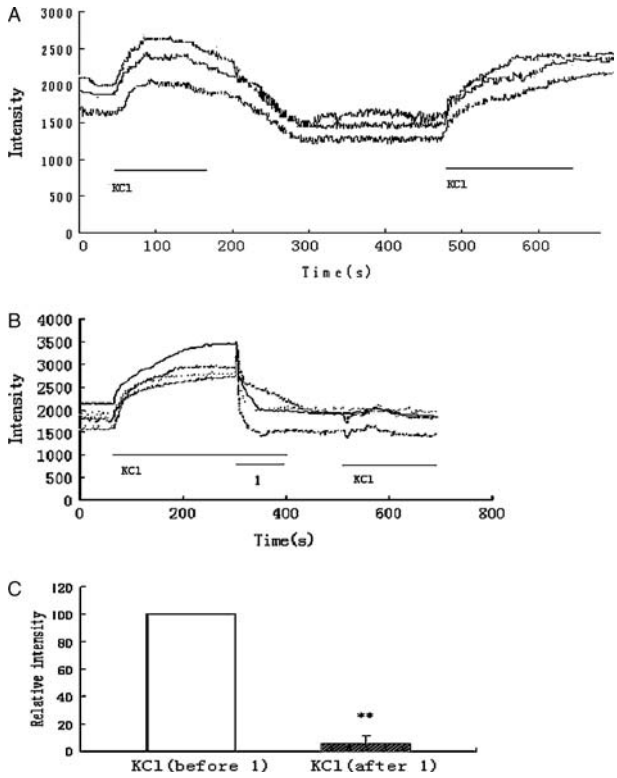

Figure 3. (A, B) Depolarization-evoked (KCl) increases of $[Ca^{2+}]_i$ in four cells in the absence or presence of 100 μM of **1**. (C) Elevation of fluorescence ratio during subsequent stimulation with 60 mM KCl in controls and **1**-treated cells, the values of KCl (after **1**) represent the percentages by that of KCl (before **1**) responses, data come from at least four different experiments and 20 cells. $^{**}P < 0.01$ compared with KCl (before **1**)-treated group.

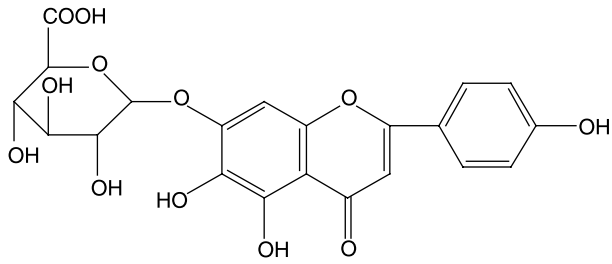

Figure 4. The structure of scutellarin (**1**).

showed nearly the same phenomenon as the results from the plant alkaloid tetrandrine conducted by other researchers.[12] Moreover, we also found a significant inhibition of **1** to sarcoplasmic reticulum Ca^{2+} ATPase activity in isolated sarcoplasmic reticulum proteins from the thoracic aortic smooth muscle cells of rat (data not shown). Third, **1** treatment irreversibly abolished the Ca^{2+} influx induced by KCl. This phenomenon was not observed in the case of phenylephrine, which is supposed to induce Ca^{2+} influx through non-voltage-dependent Ca^{2+} channels and induce a repeatable Ca^{2+} influx under **1** treatment (data not shown). These results strongly suggest an inhibitory effect of **1** on voltage-dependent Ca^{2+} channels in thoracic aortic smooth muscle cells of the rat. Consistent with our suggestion, using patch clamp technique, other researchers also found that **1** inhibited Ca^{2+} influx in cardiac muscle cells of guinea pig.[13] Therefore, we concluded that **1** increases $[Ca^{2+}]_i$ by the blockade of sarcoplasmic reticulum Ca^{2+} ATPase, and it may also block voltage-dependent Ca^{2+} channels in the thoracic aortic smooth muscle cells of rats.

3. Materials and methods

3.1 Animals

Male Wistar rats were supplied by the Experiment Animal Center of Sun Yat-Sen University in Guangzhou, China. All procedures complied with the standards for the care and use of animal subjects in the Guide for the Care and Use of Laboratory Animals (issued by the Ministry of Science and Technology of China, Beijing). All rats weighing 200–250 g were accepted.

3.2 Drugs and chemicals

Thapsigargin (Sigma, USA) was dissolved in DMSO and stored at −20°C until use. Compound **1** (Figure 4) was received from Yunnan Plant Pharmaceutical Co., Ltd. China (purity >98.5%). All solutions were made freshly before use. Fluo-3 acetoxymethyl ester (fluo-3 AM), KCl, papain, collagenase (type I), elastase (type II), trypsin inhibitor and BSA were obtained from Sigma. All other chemicals and reagents were of analytical grade and obtained from the usual commercial sources.

3.3 Cell preparations

Male Wistar rats weighing 200–250 g were housed in separate cages and treated according to the standards for the care and use of animal subjects stated in the Guide for the Care and Use of Laboratory Animals (issued by the Ministry of Science and Technology of China, Beijing). Single thoracic VSMC was enzymatically isolated from the thoracic aortas as described previously with a little modification.[6,7] Briefly, the rats were anesthetized by the intraperitoneal injection of pentobarbital sodium (60 mg/kg body weight) and decapitated. Thoracic aortas were carefully dissected and quickly placed in

low-Ca^{2+} Tyrode–Hepes (T–H) solution (in mM: NaCl, 137; KCl, 2.7; $MgCl_2$, 1.0; $CaCl_2$, 0.18; glucose, 5.6 and Hepes, 4.2; pH adjusted to 7.35 with NaOH) aerated with 95% O_2 and 5% CO_2. The aortas were then cleaned of adherent tissue and immersed in T–H solution contiguously aerating with 95% O_2 and 5% CO_2 for 120 min. After that, the aortas were cut open longitudinally and the endothelium was scraped off slightly. The media layer of thoracic aortas were transferred to a vial containing 1 ml of the same solution with collagenase (1 mg/ml), elastase (0.5 mg/ml), papain (1 mg/ml), trypsin inhibitor (1 mg/ml) and BSA (2 mg/ml) for 60 min at 37°C. The tissue was then digested in 1 ml of the same digestive juice for an additional 40 min. After completion of digestion, the tissue was washed twice in the low-Ca^{2+} T–H solution and transferred in K–B solution (in mM: potassium glutamate, 50; KOH, 20; KCl, 40; Taurine, 20; $MgCl_2$, 3.0; glucose, 10; EGTA, 0.5 and Hepes, 10; pH adjusted to 7.35 with HCl). Single cells were dispersed by gentle agitation using a wide-pore glass pipette. The dispersed single cells were affixed to cover slips and stored at 4°C and used within 8 h. VSMC were studied by an inverted phase contrast microscope and identified by positive immunocytochemical staining against α-smooth muscle actin as described previously.[8]

3.4 Ca^{2+} measurement and imaging

VSMC affixed to cover slips were loaded with 4 μM Fluo-3 AM in Hepes-balanced sodium (HBS) buffer solution (in mM: NaCl, 145; KCl, 3.0; $MgCl_2$, 1.0; $CaCl_2$, 2.0; glucose, 10; Hepes, 10 and 1% BSA; pH adjusted to 7.4 with NaOH) for 30 min at room temperature in the dark. After being loaded, the cells were placed in a chamber (0.5 ml in volume) on the stage of an inverted microscope and superfused with the bath HBS solution. The Fluo-3 fluorescence was recorded using a confocal laser scanning system (Olympus, Tokyo, Japan) equipped with an Olympus FV500 inverted microscope with a 20×, 1.4 NA objective. Fluo-3 AM was excited at 488 nm with an Argon laser. The emitted light was collected above 526 nm. At least five cells were examined in every test. For the Ca^{2+}-free test, Ca^{2+} was omitted and 1 mM EGTA was added in the solution. The fluorescence images were recorded every 5 s and analyzed frame by frame with the data acquisition being 800 × 600 pixels. Intracellular Ca^{2+} was expressed as a ratio of fluorescence intensity relative to basal fluorescence (F presents the peak value of fluorescence intensity after being activated by an agonist and F_0 presents the value of fluorescence intensity under resting conditions; $\geq F = F - F_0$). The increase in the fluorescence intensity of Fluo-3 AM is proportional to the rise in the level[9,10] of intracellular Ca^{2+}.

3.5 Statistical analysis

All values were expressed as mean ± SEM. Data analysis was performed using worksheet program, Microsoft Excel t-test. $P < 0.05$ was considered statistically significant.

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