Sanguinarine Rapidly Relaxes Rat Airway Smooth Muscle Cells Dependent on TAS2R Signaling

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INTRODUCTION

Asthma is a chronic airflow obstruction disease characterized by airway inflammation, airway remodeling and airway hyper-responsiveness (AHR) affecting more than 300 million people worldwide. 1-3 AHR is instigated by the hypercontractility of airway smooth muscle cells (ASMCs) which decreases airway lumen diameter and thereby leads to airflow obstruction.4-6 Therefore, exacerbation of acute airway narrowing evoked by some irritants is dangerous and even deadly.5,7 Currently, one of the leading treatments for asthma is focusing on relaxing ASMCs, which heavily relies on the β2-adrenergic receptor (β2-AR) agonists.8 However, more than half of asthmatic patients do not receive adequate asthma controls with these currently available muscle relaxation therapies.6,9 In addition, long term administration of β2-AR-agonists decreases β2-AR expression and agonist-stimulated generation of cAMP, which leads to insensitivity and desensitization of these β2-AR-agonists and has been associated with tachyphylaxis.7,8 Even more, increased bronchial hyper-reactivity and adverse effects have been observed in various clinical trials of β2-AR agonists.9 Therefore, it is highly desirable to develop new bronchodilators that can act through a different pharmacologic principle.6

As a potential candidate, bitter taste receptors (TAS2Rs) have been considered as novel targets to relax ASMCs. TAS2Rs are mainly expressed in the taste bud that is originally considered to prevent the ingestion of toxic substances.10,11

However, significant advances have been made in the past decade in mapping the distribution and the physiological roles of TAS2Rs in extra-oral system.12-16 In particular, TAS2R10, 14 and 31 are the most highly expressed subtypes in ASMCs.17 Furthermore, it is found that TAS2R agonists have therapeutic potential in asthma in cell-based assays and preclinical animal models.18,19 For example, TAS2R agonists such as chloroquine increased intracellular free calcium concentration ([Ca2+]i) in ASMCs in a Gβγ-, phospholipase Cβ (PLCβ)-, and inositol trisphosphate 3 (IP3) receptor-dependent manner, and caused relaxation of airways.7 More importantly, Robinet et al. have demonstrated that TAS2R expression, signaling, and ASMCs relaxation effects were not altered under airway inflammatory conditions.20 However, most of the well-known bitter tastants often function at mid-to-high micromolar which limits their clinical approval.21,22 Therefore, more potent bitter substances for ASMCs relaxation drugs need to be developed.

Interestingly, many folk medicines have been reported to function to calm panting and suppress coughing,23,24 which may function by regulating the contractility of ASMCs.25 Additionally, structurally diverse bitter substances have been clarified from these folk medicines,26 providing a gold mine for screening potent ASMC relaxants. Sanguinarine (SA) is mainly found in the bloodroot (Sanguinaria canadensis L., Papaveraceae), which has various medical functions such as anti-inflammation27,28 and anti-tumor.29 It has also been used for the treatment of respiratory diseases in a small dose in folk medicines such as asthma.30-32 Furthermore, according to...
an analysis of the gene expression profile of asthma patients, SA has a potential usage in the clinical treatment of allergic asthma.\(^{33}\) Interestingly, in a more recent study, Park et al.\(^{34}\) found that SA blunts the contraction force of Schlemm’s canal endothelial cells. Additionally, it has been shown that SA can rapidly cause relaxation in blood vascular smooth muscle\(^{35}\) and can suppress contractions of intestinal smooth muscle cells.\(^{36}\) We also found that low concentration (<1 µM) of SA decreased the hyper-contractility of cultured rat AMSCs after long term treatment for 24 h.\(^{37}\) However, it is unknown whether SA can rapidly relax ASMCs and the possible underlying mechanisms.

In this study, we investigated the rapid relaxation role and mechanisms of SA on rat ASMCs with emphasis on TAS2Rs signaling in vitro. Results indicate that SA exerted rapid relaxation function dependent on the TAS2R signaling pathway.

**MATERIALS AND METHODS**

**Materials**

SA (Fig. 1A, >98% purity confirmed by HPLC, #B21412) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Fluo-4/AM (#93596), transferrin (#T8158), and insulin (#91077C) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). All other reagents were purchased from Fisher Scientific unless noted otherwise.

**Primary Culture of ASMCs** Primary ASMCs were isolated from the Sprague-Dawley (SD) rats according to the method described previously\(^{38,39}\) which was approved by the Committee of Changzhou University on Studies Ethics. The rats (180–220 g; 6–8 weeks) were purchased from Cavens Lab Animal Co., Ltd. (Changzhou, China) and raised in pathogen-free conditions at approx. 25°C, provided with a 12 h light/dark cycle and free access to food and water. The use of animals in experiments is regulated under the Institutional Guidelines for Animal Care and Use Committee of the Changzhou University. The rats were first treated with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). The tracheas were then harvested and the connective tissues were detached. After cutting longitudinally, they were enzymatically dissociated with 0.25% trypsin−0.02% ethylenediaminetetraacetic acid (EDTA) in Hanks’ balanced salt solution (in mM: 5 KCl, 0.3 KH\(_2\)PO\(_4\), 138 NaCl, 4 NaHCO\(_3\), 0.3 Na\(_2\)HPO\(_4\), and 1.0 glucose) for 20 min at 37°C. Dissociated cells were centrifuged, resuspended in DMEM supplemented with 10% FBS and antibiotics, seeded into culture flasks, and incubated at 37°C in humidified air containing 5% CO\(_2\). ASMCs were identified with an anti-α-SMA antibody (#ab119952, Abcam). These cells showed typical “hill and valley” appearance under phase-contrast microscopy and physiological response to agonists. The cells at a passage of 3–10 were used for experiments.

**Treatment of Cultured ASMCs with Sanguinarine Inhibitors**

ASMCs were firstly cultured in DMEM with 10% FBS for 24 h, and then the medium was replaced with fresh serum-free DMEM (supplemented with 5 ng/mL transferrin and 5 ng/mL insulin). After cultured for another 12 h, the cells were treated with 0.005, 0.05, or 0.5 µM SA, which has been shown in our previous study to cause no toxic effect on the ASMCs.\(^{40}\) To block TAS2R signaling, the cultured ASMCs were separately treated with 6-methoxyflavonone (6-Met, TAS2R inhibitors, 500 µM), gallein (40 µM, an inhibitor of G\(_o\)), U73112 (an inhibitor of PLC\(\beta\), 20 µM), and 2-APB (an IP\(_3\) receptor antagonist, 40 µM) for 0.5 h. To block BK channels, ASMCs were pretreated with iberiotoxin (IbTx, 300 nM) for 0.5 h. Subsequently, they were treated with SA. A final dimethyl sulfoxide (DMSO) concentration of 0.1% (v/v) was used to avoid toxic effects on the cells.\(^{12}\)

**Assessment of Cell Stiffness of Cultured ASMCs**

Cell stiffness of cultured ASMCs was measured by optical magnetic twisting cytometry (OMTC) as described previously.\(^{36}\) In brief, ASMCs cultured on type I collagen-coated dishes (96-well plate, Immunon II) were first labeled with Arg-Gly-
Asp (RiG)-coated ferrimagnetic microbeads for 20 min and were washed with DMEM to remove unbound beads. Then the microbeads were magnetized horizontally with a 1000-Gauss pulse and twisted in a vertically aligned homogeneous magnetic field (20 Gauss) at 0.75 Hz, resulting in rotation and displacement of the bead. Cell stiffness was expressed as the ratio of twisting torque to bead displacements. In this study, the cells were first measured for 60 s to achieve baseline stiffness. Then SA was added to the cells with or without inhibitors for TAS2R signaling, and the cells were continuously measured for up to 300 s. The dynamics response of ASMCs to SA decreased cell stiffness (Fig. 1H), indicating that a lower dose of SA (0.5 μM) elicited a rapid relaxation response of cultured ASMCs similar to that of ISO (5 μM). To ascertain whether SA functions through activating the phosphodiesterase inhibitor IBMX (10−5 M) in PBS and centrifuged at 14000 rpm for 10 min under 4°C. Then the manufacturer’s protocol for the cAMP ELISA kit (#ab65355, Abcam) was employed. Absorbance at 450 nm was measured using a colorimetric 96-well plate reader (Infinite F50, Tecan, Zürich, Switzerland). Data are expressed as ratios of the optical density of groups treated with SA or ISO to that of vehicle control groups.

Statistical Analysis
Statistical analyses were performed by using GraphPad Prism (Graph Pad Software, La Jolla, CA, U.S.A.). Unless stated otherwise, data are reported as means ± standard error of the mean (S.E.M.), and n represents the number of samples. Results were analyzed by one-way ANOVA, followed by post hoc Multiple Comparisons. The significance level was set at p < 0.05.

RESULTS
Effect of Sanguinarine on Cell Stiffness Generated by Cultured ASMCs
Cell stiffness can reflect the contraction/relaxation of cultured ASMCs.31 We first analyzed dynamic changes in cell stiffness of rat ASMCs in vitro in 5 min induced by SA by using OMTC (Fig. 1). As shown in Fig. 1B, SA immediately decreased cell stiffness of ASMCs in a dose-dependent manner from the baseline. The maximum relaxation response was reached approx. 40% reduction at 0.5 μM SA. To compare the efficacy of SA with ISO (5 μM, a positive ASMC relaxant), the cell stiffness response of ASMCs to ISO was also measured as shown in Fig. 1C. The results show that the 5 μM ISO also induced a 40% reduction of cell stiffness. These results imply that a lower dose of SA (0.5 μM) elicited a rapid relaxation response of cultured ASMCs similar to that of ISO (5 μM).

To ascertain whether SA functions through activating the TAS2R signaling pathway, cultured ASMCs were pretreated with TAS2R inhibitors 6-Met, Gβγ antagonist gallein, PLCβ inhibitor U73122, or IP3 receptor blocker 2-APB, and then the cell stiffness responses of cultured ASMCs to SA have evaluated again (Figs. 1D–G). These results show that the inhibition of TAS2R signaling pathway attenuated SA-decreased cell stiffness. Together, these data show that small dose of SA immediately decreased cell stiffness dependent on TAS2R signaling.

To explore whether the SA-decreased cell stiffness is mediated by BK channels, cultured ASMCs were pretreated with 300 mM IbTx for 30 min to block BK channels and then treated with 0.5 μM SA. The results showed that IbTx partially abolished the SA-decreased cell stiffness (Fig. 1H), indicating that...
the opening of BK channels was indeed involved in mediating the SA-induced relaxation of ASMCs.

**Effect of Sanguinarine on Traction Force Generated by Cultured ASMCs**
Traction force is equivalent to cell contractile force and the modulation of traction force is often the main therapeutic strategy of bronchodilators for ASMCs. 42,43) We then tested whether SA also decreases the traction force of ASMCs by using traction force microscopy (Fig. 2). The representative phase-contrast image, the displacement of the magnetic beads and the traction force field of single ASMC are shown in Fig. 2A left, middle, and right, respectively. These results show that cells generated higher traction force at the two ends of the cells. The decreased traction force upon stimulation with ISO is shown in Fig. 2B. Compared with the control group, 0.5, 5, and 50 μM ISO induced a significant decrease in the traction force with 123 ± 13 Pa (p < 0.01), 160 ± 37 Pa (p < 0.01), and 246 ± 39 Pa (p < 0.01), respectively. As shown in Fig. 2C, the traction force generated by cultured ASMCs was also decreased with 43 ± 21 Pa (p < 0.05), 136 ± 12 Pa (p < 0.01), 183 ± 24 Pa (p < 0.01) when cells were treated with 0.005, 0.05, and 0.5 μM SA, respectively (p < 0.05). These results indicate a rapid decrease in traction force of cultured ASMCs treated with SA, although their maximum effects are lower than that of ISO.

To further test whether SA decreases the traction force of cultured ASMCs via activating TAS2R signaling pathway, cells were pretreated with 6-Met (500 μM), gallein (40 μM), U73122 (20 μM), or 2-APB (40 μM) for 30 min before exposure to SA (0.5 μM). The results show that SA-mediated traction force decrease in cultured ASMCs was attenuated when TAS2R signaling pathway was inhibited (Fig. 2D). To further determine whether BK channels mediate the SA-decreased traction force, cultured ASMCs were treated with IbTx (300 nM) for 30 min before the treatment with SA. The data shown in Fig. 2D indicate that the blocking of BK channels also significantly abolished SA-decreased traction force. Combining the above-mentioned effect of SA on cell stiffness and traction force, these findings establish the rapid relaxation effect of low concentration of SA on ASMCs, which is dependent on TAS2R signaling and the opening of BK channels.

**Effect of Sanguinarine on [Ca2+]i in Cultured ASMCs**
Intracellular calcium signaling evoked by TAS2Rs agonists has been reported to initiate the relaxation of ASMCs. 17) To test whether SA evokes intracellular calcium signaling, the [Ca2+]i response of ASMCs to SA was visualized with Fluo-4/AM. Figure 3A shows the dynamic changes of ratios of fluorescence intensity in cultured AMSCs treated with SA normalized to baseline values. The results indicate that SA (0.005, 0.05, or 0.5 μM) rapidly increased the [Ca2+]i in cultured ASMCs. At very low concentration, SA (0.005 μM) initiated a weak response in [Ca2+]i, However, with the concentration increasing to 0.05 and 0.5 μM, SA evoked a dramatic increase in [Ca2+]i, and the peak value of the [Ca2+]i response reached to approx. 2.5-fold of the baseline values. These data suggest that SA evoked the [Ca2+]i response via a dose-dependent manner.

Consistent with the data of cell stiffness and traction force, SA-increased [Ca2+]i in ASMCs was also attenuated by the antagonists of the TAS2R signaling pathway (Fig. 3B). The results show that maximum response of ASMCs in [Ca2+]i to 0.5 μM SA decreased from about 2.5-fold to 1.75-, 1.5-, 1.2-, 1.1-fold when the cells were pretreated with 2-APB, U73122, 6-Met, and gallein, respectively. These data suggest that the SA-induced Ca2+ signaling response in ASMCs was dependent on the activation of TAS2Rs.

**Effect of Sanguinarine on the cAMP Expression in Cultured ASMCs**
The increase of cAMP triggered by β2-agonists such as ISO can initiate the relaxation of ASMCs. 44) To test whether SA functions via the increase in cAMP, the cAMP level inside ASMCs treated with ISO or SA were measured with ELISA assay. We first detected the intracellular cAMP level in ASMCs after treated with 5 μM ISO for 10, 20, and 40 min. The data indicate that the intracellular cAMP level in ASMCs increased in response to ISO treatment in a time-dependent manner, reaching maximum at 30 min (Fig. 4A). In contrast, the cAMP level inside ASMCs treated with 0.5 SA at 10, 20, 30, and 40 min did not change significantly over the time course (Fig. 4B). Then, we compared the cAMP level inside ASMCs treated with ISO or SA at the same time.
point of 30 min but different doses. As shown in Figs. 4C and D, ISO increased the cAMP level inside ASMCs in a dose-dependent manner, whereas SA did not change the cAMP level inside ASMCs, although they both initiated relaxation of ASMCs. These results indicate that SA functioned independent of an increase of cAMP.

Sanguinarine Decreased the Gene Expression Contributing to the Contractile Phenotype Since we observed relaxation effect of SA on ASMCs, we wished to ascertain whether SA regulates the expression of protein correlated with the contractility of ASMCs for long term treatment. To do so, we examined the mRNA of calponin, α-SMA, and histamine receptors 1 and 2 in ASMCs treated with SA for 24 h. The results show that 0.05 and 0.5 μM SA significantly decreased the mRNA expression in cultured ASMCs both for calponin (Fig. 5A), α-SMA (Fig. 5B), histamine receptors 1 and 2 (Figs. 5C, D). These results indicate that SA may decrease the contractile activity of ASMCs to agonists.

DISCUSSION

In this study, we found that low concentration of SA (i.e., below 1 μM) was able to rapidly relax ASMCs in vitro, as evidenced by an immediate reduction of cell stiffness/traction force and an increase in [Ca^{2+}], with a dose-dependent manner. Inhibition of TAS2R signaling molecules such as TAS2Rs, Gβγ, PLCβ, and IP3 receptors attenuated the above-mentioned responses of cultured ASMCs, suggesting that SA induced relaxation of ASMC dependent on TAS2R signaling.

Biomechanical properties including cell stiffness and traction force can be used to ascertain the contraction/relaxation response of cultured ASMCs to various stimulation. In this study, OMTc was used to evaluate cell stiffness before and after the treatment of SA and ISO. We found that SA relaxed ASMCs with similar magnitude induced by ISO. We also used traction force microscopy to assay the traction force response of cultured ASMCs to SA and ISO. We found that SA also rapidly decreased the traction force of ASMCs but with greater capability when compared to ISO.

TAS2Rs have been identified as a new target for relaxing

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Fig. 3. SA Increased [Ca^{2+}], in Cultured Rat ASMCs in a TAS2R Signaling-Dependent Manner Visualized with Fluo-4/AM
(A) SA evoked [Ca^{2+}], responses with a dose–response manner; (B) The [Ca^{2+}], response to SA was ablated by inhibitor for TAS2Rs (6-Met, 500 μM), Gβγ (gallein, 40 μM), PLCβ (U73122, 20 μM), and IP3 receptor (2-APB, 40 μM). Results are means ± S.E.M. from three experiments. n = 20–30 cells.

Fig. 4. The Intracellular cAMP Level in Cultured Rat ASMCs Measured with ELISA
(A) Time course of cAMP level inside ASMCs treated with 5 μM isoproterenol (ISO); (B) Time course of cAMP level inside ASMCs treated with 0.5 μM sanguinarine (SA). (C) The cAMP level inside ASMCs treated with 0.05, 0.5, and 5 μM ISO for 30 min; (D) The cAMP level inside ASMCs treated with 0.005, 0.05, and 0.5 μM SA for 30 min. All results are means ± S.E.M. from three experiments. *p < 0.05 versus control groups; **p < 0.01 versus control groups.

Fig. 5. The mRNA Expression of Calponin (A), α-SMA (B), Histamine Receptor 1 (His R1) and Histamine Receptor 2 (His R2) in Cultured Rat ASMCs Exposed to SA for 24 h
Results are means ± S.E.M. from three experiments. *p < 0.05 versus control groups; **p < 0.01 versus control groups.
ASMCs. Bitter substances from folk medicines may comprise a new class of bronchodilators for treating asthma. Unfortunately, although thousands of bitter substrates have been identified, many of them are not extremely potent in terms of relaxing ASMCs. In this study, we found that SA can relax ASMC below 1 µM. Other well-known bitter substances investigated so far such as chloroquine or saccharine need ten to hundred micromolar concentration to induce this similar response. Therefore, SA seems to be a highly potent relaxant for contracted ASMCs.

Since the contraction of ASMCs is always triggered by the increase in [Ca\(^{2+}\)], and caused by the following excitation-contraction coupling, the relaxation of ASMCs can be realized by opposing this increased [Ca\(^{2+}\)], or by decreasing the Ca\(^{2+}\) sensitivity. This can be mediated by the activation of atypical G\(_i\)-coupled β\_adrenoceptors, which in turn increases the cAMP level via the activation of adenylyl cyclase and the following activation of protein kinase A (PKA) in ASMCs, as PKA can further reduce the [Ca\(^{2+}\)], and the Ca\(^{2+}\) sensitivity. However, we found no evidence for SA-induced increase in cAMP in cultured ASMCs. This result indicates that SA-mediated ASMC relaxation was independent of cAMP. Instead, the SA-induced effect on ASMCs was more similar to the G\(_i\)-coupled TAS2Rs-mediated relaxation.

G\(_i\)-coupled TAS2Rs have been reported to trigger an increase in [Ca\(^{2+}\)], via the activation of PLC\(\beta\) and thus the relaxation of ASMCs. In this study, we found that SA-elicited relaxation and [Ca\(^{2+}\)] responses of ASMCs were ablated by inhibiting the TAS2R signaling, confirming the relaxation effect of SA on ASMCs was dependent on the [Ca\(^{2+}\)], elicited by the activation of TAS2Rs, which is consistent with chloroquine and saccharine-evoked TAS2Rs signaling pathways.

However, it is still unknown how activated Ca\(^{2+}\) signaling following the activation of TAS2Rs triggers the relaxation of ASMCs. Deshpande et al. suggest that Ca\(^{2+}\) increase opens BK channels and thus induces membrane hyperpolarization and the relaxation of ASMCs. However, Zhang et al. reported that bitter tastants-mediated relaxation of ASMCs may not depend on BK channels. Although whether BK channels mediate the relaxation of ASMCs is still a conundrum, Bradley et al. definitely show that contraction of ASMCs can be abolished by novel potent BK channel openers. In this study, the results show that specific inhibition of BK channels did partially inhibit the SA-mediated relaxation in ASMCs, confirming to a certain extent the BK channels involve in this relaxation process. Beside the opening of BK channels, the inhibition of voltage dependent L-type Ca\(^{2+}\) channels and non-selective cation channels have also been suggested to be involved in TAS2Rs-triggered relaxation of ASMCs. Their roles in SA-mediated transient relaxation of ASMCs may also need to be carefully studied in the future.

Like most bitter substances, SA has been widely reported to have anti-inflammatory effects in vitro and in vivo. Additionally, it is shown that SA inhibits some enzymatic activities such as Rac1b and PKC, and the response of receptors such as vasopressin V1 receptor and γ-aminobutyric acid (GABA)A, which all regulate the cytoskeleton dynamics and promote the contraction of ASMCs in asthma. In this study, we also found that SA significantly inhibited the expression of contractile proteins such as calponin and α-SMA in ASMCs, which may attenuate the contractility of ASMCs.

Therefore, SA may contribute to asthma therapy in a variety of mechanisms.

In this study, we mainly verified the rapid relaxation effect of SA on rat ASMCs in vitro. Considering the expression profiles of TAS2Rs are different between rats and humans, we need to assay the effects of SA on human ASMCs in vitro. Although cell culture model is simple for understanding the mechanisms, we also still need to evaluate the above-mentioned effect of SA in vivo such as asthma pre-clinical models to ascertain the value of SA as a useful bronchodilator for asthma patients. Additionally, the current findings were limited to the effect of SA on the basal tone of ASMCs. In the pathological conditions, however, such as those associated with asthma, the ASMCs are always already contracted by stimulation of diverse bronchoconstrictors such as histamine, acetylcholine, and leukotriene. Thus, it is important to further explore whether SA can rapidly relax the pre-contracted ASMCs as a potent therapeutic drug agent for relief of acute airway constriction.

It is worthy to note that SA is known to cause inotropic effect in guinea pig atria due to its inhibitory effect on the Na\(^{+}\), K\(^{-}\)-ATPase in myocardium, suggesting cytotoxic effect of SA on cardiac cells. This may be a risk factor to be evaluated carefully in future study of clinical applications of SA in asthma therapy. Such possible cytotoxicity of SA on cardiac cells should, however, not be a major concern with the use of SA for ASMC relaxation for two reasons. First, the concentration of SA for effective relaxation of ASMCs may be ten times lower than the reported IC\(_{50}\) for inhibition Na\(^{+}\), K\(^{-}\)-ATPase in myocardium (0.5 versus 6 µM). Second, as a bronchodilator SA in clinical practice is most likely to be directly atomized into airways just as in the way the most other bronchodilators are administrated. Therefore, SA would probably be confined largely to cause local effect on the respiratory cells instead of reaching the distant cardiac cells for adverse effect.

**CONCLUSION**

Taken together, a low concentration of SA rapidly decreased the cell stiffness and traction force and immediately increased the intracellular calcium signaling in cultured ASMCs both dependent on activating TAS2R signaling. SA also dose-dependently decreased the expression of contractile proteins such as calponin and α-SMA after a long term of treatment. These results suggest that SA may have potential to be developed as novel bronchodilators for the treatment of asthma.

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**Conflict of Interest** The authors declare no conflict of interest.
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