Bitter Taste Receptor Agonist (Quinine) Induces Traction Force Reduction and Calcium Flux Increase in Airway Smooth Muscle Cells from Ovalbumin-Sensitized and Challenged Rats

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Abstract: Recently, bitter taste receptors (TAS2Rs) have been found in the lung, which can be stimulated with TAS2R agonist such as quinine to relax airway smooth muscle cells (ASMCs) via intracellular Ca^{2+} signaling generated from restricted phospholipase C activation. This provides a promising new therapy for asthma because enhanced contractility and impaired ability of relaxation of the ASMCs within the bronchial wall of asthmatic patients are thought to be ultimately responsible for airway constriction in asthma. However, further study is required for characterization of the effect of TAS2R agonist on the mechanical behaviors of ASMCs, in particular the traction force generation and associated mechanism in asthma model. Here, we sensitized Sprague Dawley rats with ovalbumin (OVA) for up to 12 weeks to simulate chronic asthma symptoms. Subsequently, we isolated ASMCs from these rats, and studied the traction force and intracellular Ca^{2+} signaling of the cells with/out treatment of quinine hydrochloride, a well-known TAS2R agonist. The results demonstrated that quinine hydrochloride relaxed the ASMC in a dose dependent manner. It also evoked dose-dependent increase of intracellular calcium ($[Ca^{2+}]_i$) in the ASMCs. Perhaps more importantly, the quinine-induced traction force reduction and Ca^{2+} flux increase were correlated. Taken together, our findings indicate that TAS2R agonists (*e.g*. quinine hydrochloride) could reduce the ability of ASMCs to generate traction force via activation of the intracellular calcium signaling, which may contribute as one of the mechanisms for TAS2R agonist-induced ASMC relaxation. This provides additional evidence to support TAS2R agonists as a new class of compounds with potential in treatment of chronic asthma.

Keywords: Asthma, Smooth muscle, Bitter taster receptor, Relaxation, Cell traction, Calcium signal.

1. INTRODUCTION

Asthma is a common chronic airway disease that affects about 300 million individuals worldwide, and is characterized by airway inflammation, airway tissue remodeling and airway hyperresponsiveness (AHR) [1, 2]. Although airway inflammation and tissue remodeling are important in the pathogenesis of asthma, AHR is ultimately responsible for the excessive airway narrowing that leads to difficulty or even complete loss of breathing during asthma attack. The mechanisms of AHR remain largely unexplained. However, it can be certain that acute narrowing of the airway lumen is caused by contraction of the airway smooth muscle cells (ASMCs) [3-5].

Deshpande *et al*. recently found that cultured ASM cells express G-protein-coupled bitter taste receptors (TAS2Rs) [6], a class of proteins long thought to be expressed only in the specialized epithelial cells in the taste buds of the tongue that allow organisms to avoid harmful toxins and noxious substances characterized by bitterness [7-10]. They found that bitter agents appeared to increase intracellular Ca^{2+} concentration to a level comparable to that produced by bronchoconstrictors such as those for histamine, acetylcholine and bradykinin [11], which should have led to smooth muscle contraction [12], but unexpectedly found that these agents are bronchodilators instead. Moreover, bitter taste receptor agonist cause greater ASM relaxation *in vitro* than β_2 adrenergic agonists, the most commonly used bronchodilators to treat asthma and COPD [6, 13]. However, it remains to be studied the nature of ASMC relaxation induced by TAS2R agonist and the associated mechanism in the context of treatment for asthmatic symptoms.

To study that, we isolated bronchial ASMCs from the rat models of chronic asthma and cultured them *in vitro*. Subsequently, we measured the ASMCs for traction force generated by the cell and intracellular

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calcium flux with/out stimulation by quinine hydrochloride, a well-known TAS2R agonist. We found that traction decreased and intracellular calcium flux increased as the dose of quinine hydrochloride increased. Furthermore, the quinine-induce traction force reduction and calcium flux increase seemed to be correlated. These findings demonstrated that TAS2R agonist reduced traction force generated by ASMC via stimulation of intracellular calcium flux, thus providing one mechanism through which bitter agents relax ASMCs. This may be important for understanding of TAS2R mediated airway dilation and the development of a new class of TAS2R-agonist based compounds for potential therapy of chronic asthma.

2. MATERIALS AND METHODS

2.1. Materials

Cell culture reagents and collagen Type I was purchased from Sigma–Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, NY). All other reagents were obtained from Fisher Scientific (Newark, DE), unless noted otherwise.

2.2. Experimental Animals

Sprague Dawley (SD) rats were purchased from and maintained in a specific pathogen free environment in Cavens Lab Animal Co. Limited (Changzhou, China), which is an authorized supplier of experimental animals for medical research. All animal experiments were performed according to institutional guidelines for Animal Care and Use Committee of the Changzhou University of China.

2.3. Allergen Sensitization and Challenge of Rat as Chronic Asthma Model

Female SD rats, 6 to 8 weeks old, were sensitized and challenged with OVA as described [14, 15]. Briefly, SD rats were sensitized by intraperitoneally injections of 1 mg OVA and 0.1 g Al $(OH)_3$ suspended in 1 mL saline on days 0 and 8. From day 14, the rats were persistently challenged with 1% OVA aerosol for 30 minutes and three times per week for 12 weeks. Rats receiving the same treatment schedule, but with saline instead of OVA, were used as controls.

2.4. Histopathology of the Rat Bronchial Airways

The right lung was immersed in 4% formalin overnight and dehydrated in a graded series of ethanol solutions. Tissue was embedded in paraffin. Sections were cut at 5μm thickness. Each section placed on glass slide was stained with hematoxylin and eosin (H&E) and photographed under a light microscope (Nikon, Japan) at a magnification of 100 for morphological analysis.

2.5. Isolation of the Rat Bronchial ASMCs

Primary ASMCs were isolated from the SD rats and cultured *in vitro* according to the method described previously [16]. Briefly, SD rats were i.p. injected with pentobarbital sodium (60mg/kg). The isolated tracheas were cleaned of connective tissues, cut longitudinally through the cartilage, and enzymatically dissociated with Hanks' balanced salt solution (HBSS), which consisting of (in mM): 5 KCl, 0.3 KH_2PO_4, 138 NaCl, 4 $NaHCO_3$, 0.3 Na_2HPO_4, and 1.0 glucose, containing 0.25% trypsin-0.02% EDTA solution for 20 min at 37°C. Dissociated cells in suspension were centrifuged and resuspended in DMEM supplemented with 20% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin). Cells were plated on culture flasks and grew until confluence at 37°C in humidified air containing 5% CO_2. The grown cells were verified as ASMCs by staining with antibodies against α-smooth muscle actin [17]. In this study, all the cells used were between passages 2 to 7.

2.6. Assessment of Traction Force Generated by ASMC

We adopted the technique of polyacrylamide substrate described previously [18, 19]. First, about five drops of 0.1 M NaOH were added to a cell culture dish with a glass bottom (to enable microscopy) and air-dried. Next, 3-aminopropyltrimemethoxysilane was smeared onto dried dishes with a glass pipette just as if making a peanut butter sandwich. After 5 min, the dish was washed and soaked with distilled water. The dish was immersed for 30 min in 0.5% glutaraldehyde in PBS. 10 mL of the acrylamide/bis-acrylamide mixture, containing 2% acrylamide and 0.1% or 0.25% bis concentrations, was added to the dish after being mixed with 0.2-μm-diameter fluorescent beads (F8811; Invitrogen Life Technologies, Carlsbad, CA). The gel was covered with a piece of 12-mm diameter cover glass and turned upside down to let the microbeads move to the surface of the cover glass by gravity. After 45 min, the gel formed and the circular coverslip was carefully removed. Most microbeads moved to the free surface of the gel, as determined by microscopy. The gel was ~70 μm thick, as determined by confocal

microscopy. To activate the free surface of the gel so that matrix proteins could be coated on it, 100 µL of 1 mM Sulfo-SANPAH (Pierce Biotechnology, Inc., Rockford, IL) in 200 mM HEPES were pipetted onto the gel surface. The dish was exposed to ultraviolet light for 10 min. The dish was washed with PBS twice. The gel was then coated with collagen type I and stored at 4°C before use.

Cells were seeded onto the polyacrylamide gel substrate at a density of ~2000 cells/well, incubated for 12 h to allow the cells to attach to the substrate and cultured for ≥12 h in serum-free medium. Single ASMCs were imaged with phase contrast by an inverted optical microscope, increasing concentrations of quinine hydrochloride (0.01, 0.1, and 1 mM) were added sequentially every 3 min, and fluorescent images were recorded every 40 s. After the treatments were completed, the cell was trypsinized and the cell-free bead positions were recorded as a reference point for bead displacement.

2.7. Assessment of Intracellular Calcium Flux ($[Ca^{2+}]_i$) in ASMCs

Primary rat airway smooth muscle cells were isolated and cultured as described above. Intracellular calcium signals were visualized as previously described [20, 21] using the membrane permeable $[Ca^{2+}]_i$ -sensitive fluorescent dye Fluo-4 acetoxymethyl ester (Fluo-4 AM). Cultured rats ASMCs were grown on a cell culture dish with a glass bottom (to enable microscopy). Cells were loaded with 5 µM Fluo-4 AM for 30~45 min at ~22°C. The cells were washed with Tyrode solution containing extracellular Fluo-4 AM and incubated for 15~30 min to allow complete de-esterification of cytosolic dye. The excitation wavelengths were set at 488 nm, and the emission wavelength was set at >505 nm.

2.8. Statistical Analysis

Dose response curves for $[Ca^{2+}]_i$ was analyzed by iterative non-linear least squares fitting. Results from all studies were compared using paired or unpaired two-way t-tests (depending on study design), with $P<0.05$ considered significant. When multiple comparisons were sought, an ANOVA with post-hoc t-tests was utilized with a correction for multiple comparisons. Data are presented as mean ± standard error.

3. RESULTS

3.1. Lung Histology

Figure **1** shows that airway remodeling was found in this model of chronic asthma. Rats were sensitized to OVA, and then repetitively challenged with inhaled OVA, which resulted in acute airway inflammation in lung tissue with dense peribronchiolar and perivascular infiltrates consisting of lymphocytes, eosinophils, and neutrophils. The bronchial cross-section of OVA-induced asthmatic rat model clearly shows airway smooth muscle thickening (Figure **1B**).

3.2. Immunohistochemistry Characterization of the ASMCs

Under light microscopy, both normal and asthmatic cells appeared spindle-shaped, with central oval nuclei containing prominent nucleoli, and displayed the typical “hill and valley” proliferation pattern in culture (Figure **2A**). Cells showed uniform staining for the smooth muscle–specific contractile proteins α-smooth muscle actin (SMA), indicating that these cells were ASMCs.

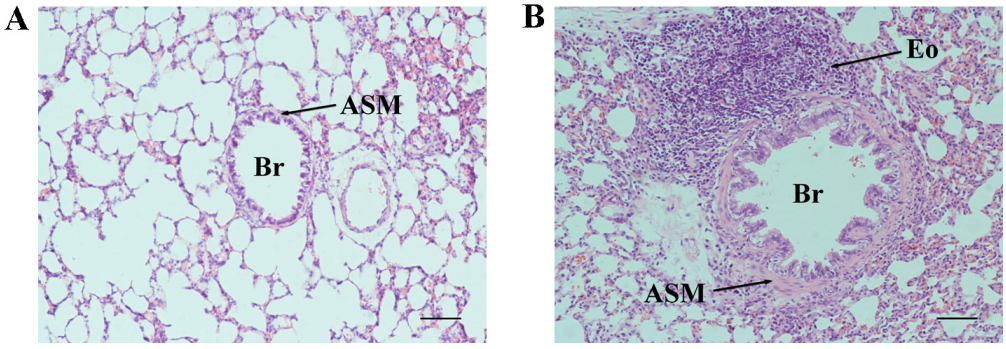

Figure 1: Photomicrographs of lung sections from either normal or OVA-sensitized and challenged rats.
(**A**) Normal lung section as control. (**B**) Lung section from OVA-challenged rat showing eosinophilic inflammation, ASM thickening in the bronchial airways as compared to the control. Lung sections were stained with hematoxylin and eosin, and the scale bar=100 µm (Br, bronchus; Eo, eosinophil; ASM, airway smooth muscle).

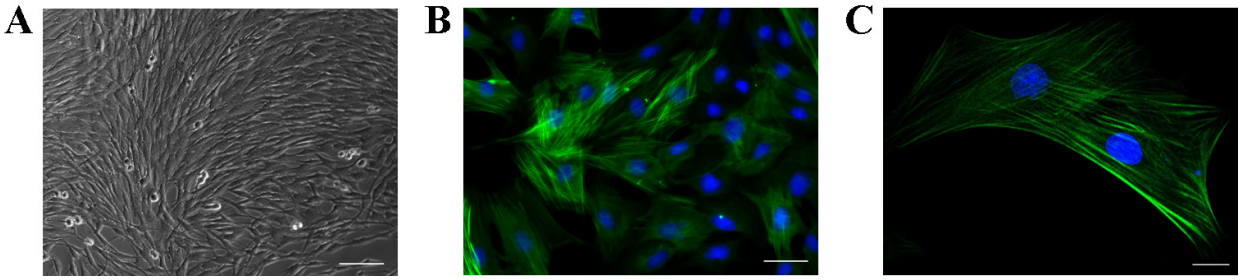

Figure 2: Characterization of the ASMCs from OVA-sensitized and challenged rat.
(**A**) Phase-contrast microscopy image of ASMCs from OVA-sensitized and challenged rat and cultured *in vitro* for 8 days. (**B**) Microscopic image of ASMCs from OVA-sensitized and challenged rat with immunohistochemical staining of α-SMA. (**C**) More detailed view of the α-SMA in the ASMCs. Nuclei were stained blue with 4, 6-diamidino-2-phenylindole (DAPI). The scale bar in panel A to C=100, 50, 20 μm, respectively.

The purity of ASMCs was confirmed to exceed 95% by α-SMA staining (Figure **2B-C**).

3.3. Traction Force Generated by the ASMCs in Response to Quinine Hydrochloride

For the present study, we used the prototypic TAS2R agonist quinine hydrochloride because it has been shown to activate the four most highly expressing TAS2Rs in human ASMCs (*i.e.*, TAS2R4/10/14/31) [6, 22].

Figure **3** shows that the traction force generated by the ASMCs from OVA-sensitized rats with treatment of quinine hydrochloride at different concentrations. The phase-contrast image shows a singular rat ASMC attached to the surface of the polyacrylamide gel coated with collagen I (Figure **3A**). And the traction

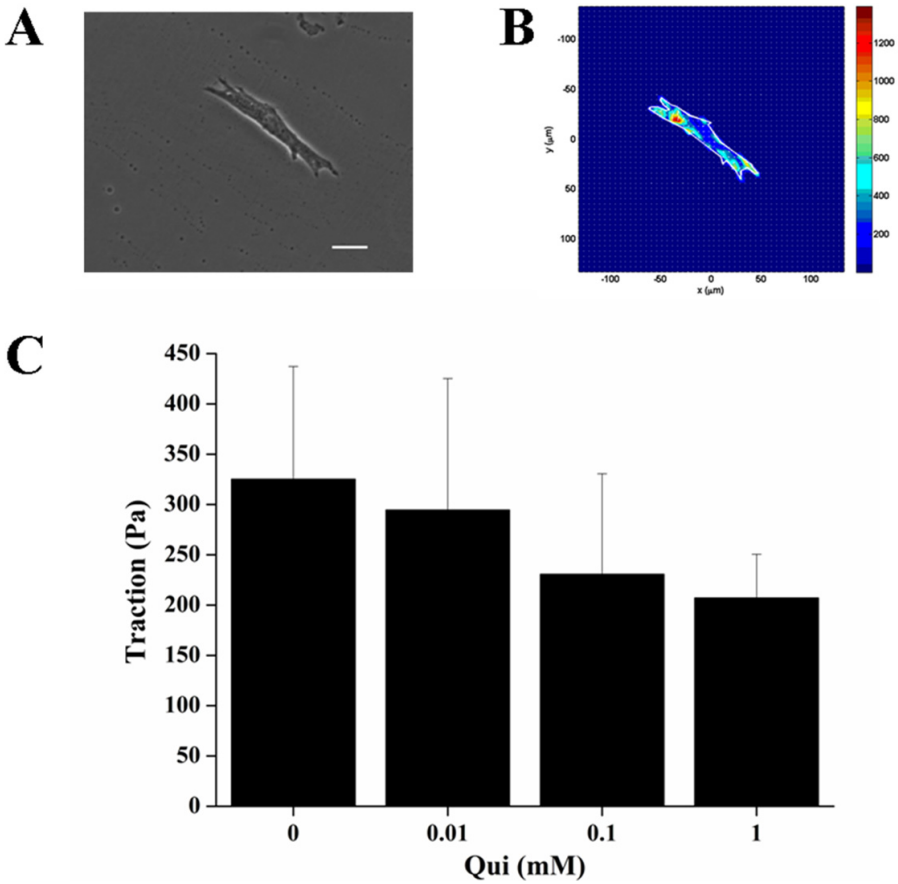

Figure 3: Traction force generated by the ASMCs from OVA-sensitized and challenged rats.
(**A**) A representative phase-contrast microscopic image of a single ASMCs cultured on polyacrylamide substrate. (**B**) A map of traction force generated by the same cell shown in (**A**), Colors show the magnitude of the tractions in Pa (see color scale). (**C**) Calculated traction force of the ASMCs versus the dose of quinine hydrochloride (Qui). Data are presented as means±SD; n=4 cells. The scale bar=20 μm.

field was calculated from the displacement field, using constrained Fourier transform traction cytometry (FTTC) (Figure **3B**). The average traction force decreased progressively with increasing dose of quinine hydrochloride, indicating that the cell's basal tone decreased as a phenomenon associated with ASMC relaxation in response to quinine hydrochloride (Figure **3C**).

3.4. Intracellular Calcium Flux in the ASMCs in Response to Quinine Hydrochloride

We examined how quinine hydrochloride affected $[Ca^{2+}]_i$, which was assessed by Fluo-4 AM, in freshly isolated ASMCs from the OVA-sensitized and challenged SD rats. Figure **4A** shows representative images of fluorescence microscopy of an ASMC before (0 s) and after 35 s of treatment with 1 mM quinine hydrochloride. It can be seen that the fluorescence that indicated the calcium flux intensified from 0 s to 35 s. Figure **4B** shows the time course of intracellular calcium flux ($[Ca^{2+}]_i$ quantified by averaging the fluorescence intensity over the entire cell) in an ASMC treated with quinine hydrochloride at concentration of 0.01, 0.1, and 1 mM, respectively, it can be seen that $[Ca^{2+}]_i$ in the ASMC responded to treatment with quinine hydrochloride in a dose dependent manner, and maximized at the time point of about 35 s. Obviously, the curve of 0.01 mM was quite different from the curves of 0.1 mM and 1 mM. It means that 0.01 mM quinine had effect on ASMCs, but reached almost the minimum effective concentration. Such responses were found to be similar in magnitude to those for known bitter taste receptor agonists.

The above results show that traction force decreased with increasing dose of quinine, whereas the intracellular calcium flux ($[Ca^{2+}]_i$) increased with increasing dose of quinine, which suggests that traction force reduction might be correlated with the $[Ca^{2+}]_i$ enhancement. To verify the correlative relationship between traction force and $[Ca^{2+}]_i$, we plotted the dose-dependent traction force versus the dose-dependent $[Ca^{2+}]_i$ as shown in Figure **5**. It can be seen that the traction force decreased as $[Ca^{2+}]_i$ increased, yielding a nearly linear but negatively correlated relationship. The results shown in Figure **5** included data from Figure **3C**, **4B**, plus data from additional experiments with other cells but not shown here.

4. DISCUSSION

Airway hyperresponsiveness and remodeling are important characteristics of asthma, and both are related to calcium levels in ASMCs. In asthma, inflammatory cells can release cytokines that in turn induce increased calcium concentration and contraction in ASMC, and ultimately airway hyperresponsiveness.

As a model system, we used ASMCs in culture. Cells in culture have certain limitations but offer the

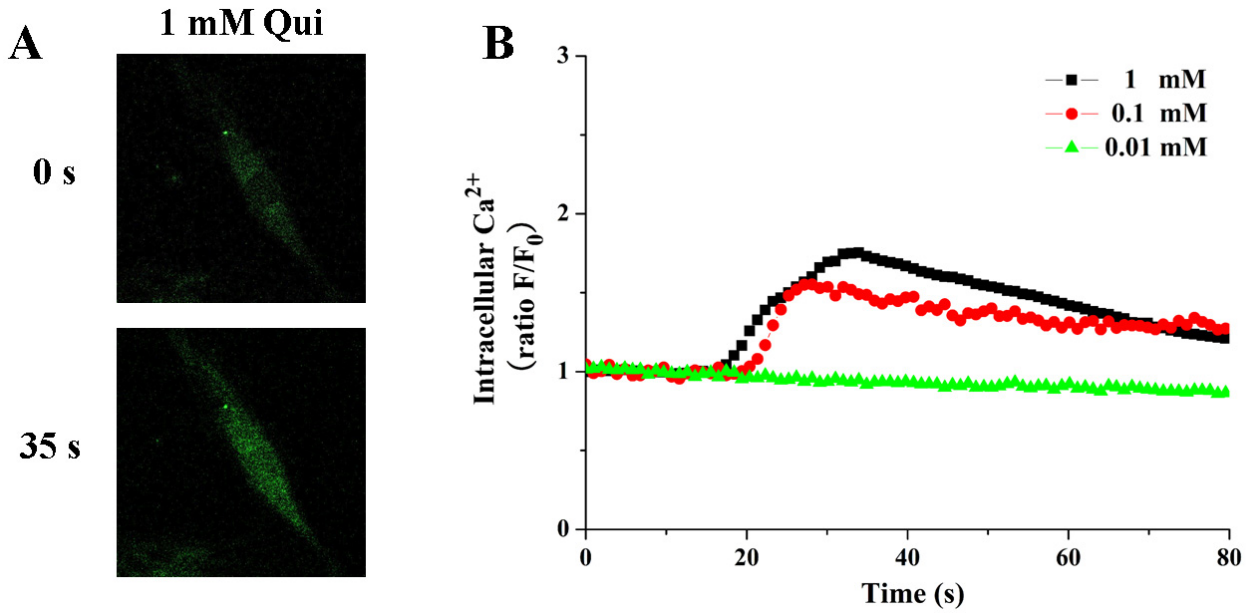

Figure 4: Quinine hydrochloride evoked $[Ca^{2+}]_i$ flux in ASMCs from OVA-sensitized and challenged rat.
(**A**) Sequential confocal images of an ASMC at 0 s and 35 s after treatment with 1 mM quinine hydrochloride. At 35 s, it clearly shows activation of $[Ca^{2+}]_i$ flux in the cell. (**B**) The dose dependent time courses of $[Ca^{2+}]_i$ transients in the ASMCs after treatment with quinine hydrochloride.

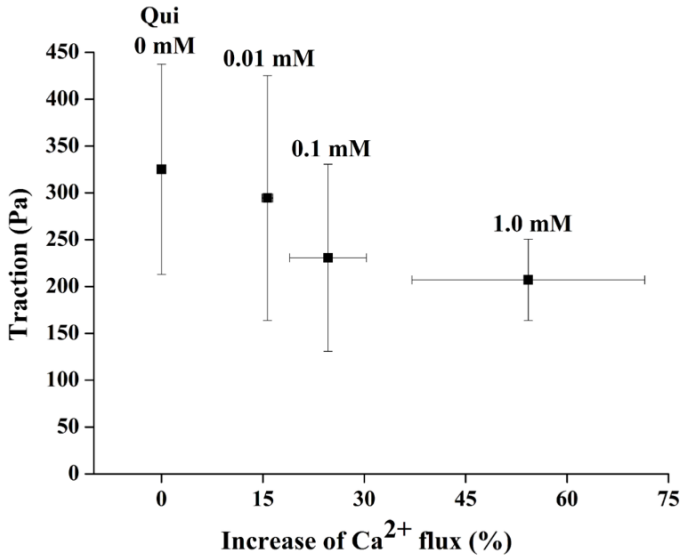

Figure 5: The dose-dependent traction force versus the dose-dependent $[Ca^{2+}]_i$.
Correlation between the traction force generated by the ASMC and the increase of calcium (Ca^{2+}) flux in the ASMC from OVA-sensitized and challenged rat in response to treatment of quinine hydrochloride (Qui) at dose from 0 to 1 mM.

advantages that ASMCs passaged in culture retain pharmacomechanical coupling to a wide panel of contractile and relaxing agonists [23-25].

Marija *et al*. have used FTTC to calculate traction force in adherent human ASMCs. They have shown that the average traction force under the cell increases in response to contractile agonists (*e.g*., histamine), and decreases in response to relaxing agents (*e.g*., isoproterenol) [26, 27]. Lin *et al*. found that compared with the controls, the traction force generated by ASMCs was marginally enhanced in the rats that had been sensitized by OVA for 12 weeks, but it was significantly greater for 4 weeks [28]. We used Fourier transform traction microscopy to measure the ASMCs for their ability to generate traction force after treatment with quinine hydrochloride at the single-cell level. The results demonstrated that traction force generated by the ASMCs decreased with increasing dose of quinine hydrochloride, which indicated relaxation of ASMCs from the rat model of chronic asthma.

In initial studies, Deshpande *et al*. found that several known bitter taste receptor agonists (such as saccharin and denatonium) evoked increased $[Ca^{2+}]_i$ in cultured human ASM cells [6]. The relaxation induced by TAS2Rs is associated with a localized $[Ca^{2+}]_i$ response at the cell membrane, which opens large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels thereby hyperpolarizing the membrane [6]. However, Zhang *et al*. found bitter tastant-induced bronchodilation may not be due to activating BK_{Ca} [13]. They suggest that TAS2R stimulation activates two opposing Ca^{2+} signaling pathways via Gβγ to increase $[Ca^{2+}]_i$ at rest while blocking activated L-type voltage-dependent Ca^{2+} channels (VDCCs) to induce bronchodilation of contracted ASM [29]. What's more, TAS2Rs undergo homologous desensitization of the $[Ca^{2+}]_i$ response in ASMCs, as well as a smooth muscle relaxation response in intact airways. Desensitization was proposed as a necessary integrative and homeostatic cellular response to the activation of GPCRs.

Here, we found that $[Ca^{2+}]_i$ increased with the dose (0.01 mM, 0.1 mM, 1 mM) of quinine hydrochloride in rat ASMCs. Perhaps more importantly, we found that the $[Ca^{2+}]_i$ signaling induced by quinine hydrochloride was negatively correlated with the traction force induced by the same agonist at the same dose range. These findings demonstrated that TAS2R agonist reduced traction force generated by ASMC via stimulation of intracellular calcium flux, thus providing one mechanism through which bitter agents relax ASMCs. This may be important for understanding of TAS2R mediated airway dilation and the development of a new class of TAS2R-agonist based compounds for potential therapy of chronic asthma.

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