Semen cassiae Extract Inhibits Contraction of Airway Smooth Muscle

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β2-adrenoeceptor agonists are commonly used as bronchodilators to treat obstructive lung diseases such as asthma and chronic obstructive pulmonary disease (COPD), however, they induce severe side effects. Therefore, developing new bronchodilators is essential. Herbal plants were extracted and the extracts’ effect on airway smooth muscle (ASM) precontraction was assessed. The ethyl alcohol extract of semen cassiae (EESC) was extracted from Semen cassia. The effects of EESC on the ACh- and 80 mM K+-induced sustained precontraction in mouse and human ASM were evaluated. Ca2+-permeant ion channel currents and intracellular Ca2+ concentration were measured. HPLC analysis was employed to determine which compound was responsible for the EESC-induced relaxation. The EESC reversibly inhibited the ACh- and 80 mM K+-induced precontraction. The sustained precontraction depends on Ca2+ influx, and it was mediated by voltage-dependent L-type Ca2+ channels (LVDCCs), store-operated channels (SOCs), TRPC3/STIM/Orai channels. These channels were inhibited by aurantio-obtusin, one component of EESC. When aurantio-obtusin removed, EESC’s action disappeared. In addition, aurantio-obtusin inhibited the precontraction of mouse and human ASM and intracellular Ca2+ increases. These results indicate that Semen cassiae-contained aurantio-obtusin inhibits sustained precontraction of ASM via inhibiting Ca2+-permeant ion channels, thereby, which could be used to develop new bronchodilators.

Keywords: Semen cassiae, aurantio-obtusin, airway smooth muscle, relaxation, contraction, ion channels

Abbreviations: ACh, acetylcholine chloride; ASM, airway smooth muscle; COPD, chronic obstructive pulmonary disease; EESC, ethyl alcohol extract of semen cassia; HPLC, high performance liquid chromatography; LVDCCs, voltage-dependent L-type Ca2+ channels; Pyr3, pyrazole 3; SOCs, store-operated channels; STIM, stromal interaction molecule; TRPC3, transient receptor potential channel 3; TRs, tracheal rings.
INTRODUCTION

Approximately 300 million and 328.6 million people suffer from asthma and COPD worldwide (Braman, 2006; Vos et al., 2012). Bronchodilators are a major group of medications for the treatment of these obstructive lung diseases (Dekhuijzen, 2015), however, they cause various side effects such as palpitations, tremor, headache, cardiovascular death (Kajioka et al., 2012), ischemic heart disease, cardiac failure, death due to life-threatening asthma attacks (Abramson et al., 2003; Cazzola et al., 2005; Hasford and Virchow, 2006; Wijesinghe et al., 2009), and desensitization (Nino et al., 2009).

We attempted to screen herbal compounds and develop new bronchodilators. *Semen cassiae* is the seed of *Cassua obtusifolia* L. and *Cassia tora* L., which are globally distributed and used as a medicine and a drink (Zhang et al., 2012). According to Chinese records, “Shennong Ben Cao Jing” (published in approximately 1602~1616, during the Ming Dynasty), *Semen cassiae* has been used as folkloric medicine to treat various diseases/disorders such as sore red swollen eyes, poor vision, headache, dizziness, diuresis, constipation, and swollen, tinea, and hepatic irrhosis ascites. Later studies have indicated that *Semen cassiae* can be used to treat other disorders, for example, neurotoxicity (Ju et al., 2010), diabetic complications (Jung et al., 2010; Fu et al., 2014; Kim et al., 2014), Alzheimer’s disease (Jung et al., 2016), allergy (Kim et al., 2015), and hypertension (Li et al., 2015). Its hypotensive effect led us to investigate whether it has relaxant action in airway smooth muscle (ASM). We found that the ethyl alcohol extract of *Semen cassia* (EESC) inhibits ASM contraction via inhibiting Ca$^{2+}$ permeant ion channels.

MATERIALS AND METHODS

Animals and Ethics Statements

Six-weeks-old male BALB/c mice were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China) and were housed in a standard animal facility. All animal use and experimental procedures were approved by the Institutional Animal Ethics Committee of the South-Central University for Nationalities. The license number is 2016-SCUEC-AEC-0030.

Human ASM

Human bronchial ASM were from lung tissue samples of transplant donors and recipients and respected tissue from subjects with lung carcinoma. The experiments adhered to guidelines and protocols approved by the Ethics Committee of the South-Central University for Nationalities, and the subjects provided signed, written informed consent.

Reagents

Nifedipine, acetylcholine chloride (ACh), pyrazole 3 (Pyr3), YM-58483, and agarose were purchased from Sigma (St. Louis, MO, United States). Fluo-4 AM was purchased from Invitrogen (Eugene, OR, United States). *Aurantio-obtusin* was purchased from Yuanye Bio-Technology (Shanghai, China). Nifedipine, Pyr3 and fluo-4 AM were dissolved in DMSO (the final concentration of DMSO ≤ 0.3% in experiments).

Plant Material and Extraction

*Semen cassiae* was purchased from Beijing TongrenTang (Wuhan, China) and was authenticated by Professor Ding-Rong Wan (College of Pharmacy, South-Central University for Nationalities). A voucher specimen was deposited at the Herbarium of the College of Pharmacy, South-Central University for Nationalities, China. *Semen cassiae* was powdered and then extracted with 70% aqueous ethyl alcohol twice. The extracts were boiled for 1.5 h, and the supernatants were collected and evaporated under reduced pressure. The dried ethyl alcohol extract of *semen cassiae* (EESC) was dissolved in DMSO.

HPLC Analysis

HPLC analysis was performed using an Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc., United States). The mobile phases consisted of 1%H$_2$PO$_4$ in H$_2$O and methanol (the ratio is 30:70). A flow rate of 5 mL/min was used and the chromatograms were monitored at 285 nm. Fractions under the peak area of 25.540 min were collected. Using the semi-preparative HPLC technique (Luo et al., 2018).

Contraction Measurements of ASM

Contraction of mouse tracheal ASM was measured in epithelium-intact and -denuded tracheal rings (TRs) as previously described, with some modifications (Kajioka et al., 2012; Zhang et al., 2014; Wei et al., 2015; Jiang et al., 2016; Liu et al., 2017). In brief, mice were sacrificed with an intraperitoneal injection of sodium pentobarbital (150 mg/kg). The tracheae were removed and transferred to ice-cold physiological salt solution (PSS) (mM): NaCl 135, KCl 5, MgCl$_2$ 1, CaCl$_2$ 2, HEPES 10, glucose 10 (pH 7.4). TRs (~5 mm) were cut from the bottom of the tracheae. Epithelium removal was achieved by gentle mechanical rubbing of the luminal surface with cotton under microscope. TRs were mounted in 10 mL organ baths containing PSS bubbled with 95% O$_2$ and 5% CO$_2$ at 37°C. A preload of 0.3 g was added. The TRs were equilibrated for 60 min and then contracted with 10$^{-4}$ M ACh three times with an interval of 30 min. After resting for 30 min, experiments were performed. Contraction in human bronchial transverse ASM strips was, similarly, measured, except for the preload was set at 0.3~0.8 g depending on the tissue size.

Contraction of mouse bronchial ASM was also measured in mouse lung slices (Bai and Sanderson, 2006; Jiang et al., 2016). Briefly, mice were euthanized, and the tracheae and lungs were exposed. The tracheae were then cannulated, and each lung was...
FIGURE 1 | EESC blocks contraction in mouse TRs. (A) ACh (100 µM) induced a sustained contraction in a mouse TR, which was blocked by EESC. The dose-inhibition curve is presented. (B) Similar experiments were performed, except that the TR contraction was induced by 80 mM K⁺. (C,D) The same experiments as above performed in epithelia-denuded TRs. These data indicate that the EESC blocks ASM contraction.
slowly inflated with a warm agarose solution (2%, 37°C, ∼1.3 mL) followed by ∼0.2 mL of air. The lungs were then cooled at 4°C to gel the agarose. Single lobes were removed and sectioned into ∼140 μm thick slices with a vibratome (VT1000S, Leica, Germany). The slices were collected and transferred to Dulbecco’s modified Eagle’s medium (DMEM) (GIBICO) and maintained in an LSM 700 laser confocal microscope (LSM 700, Carl Zeiss, Goettingen, Germany) and perfused with Hanks’ balanced salt solution (HBSS) contained (in mM) 20 mM HEPES, NaCl 137.93, KCl 5.33, NaHCO₃ 4.17, CaCl₂ 1.26, MgCl₂ 0.493, MgSO₄ 0.407, KH₂PO₄ 0.4414, Na₃HPO₄ 0.338, and D-glucose 5.56 and was adjusted to a pH of 7.4. Images under a 10× objective were acquired at a rate of 30 frames/min. The airway lumen areas were measured using Zen 2010 software (Carl Zeiss, Goettingen, Germany).

Culture of ASM

Mouse TRs were transferred in Dulbecco’s Modified Eagle Medium (DMEM, GIBICO) supplemented with 10% (w/v) fetal bovine serum (FBS, GIBICO), 100 units/mL penicillin and 100 mg/mL streptomycin, and cultured with or without EESC for 24 h at 5% CO₂ in a 37°C incubator. TRs were washed with PPS and ACh- and 80 mM K⁺-induced contractions were measured.

Isolation of Single Airway Smooth Muscle Cells

Single mouse tracheal smooth muscle cells were enzymatically isolated as previously described with some modifications (Liu et al., 2009; Wei et al., 2015). Briefly, adult male BALB/c mice were euthanized by intraperitoneal injection of sodium pentobarbital (150 mg/kg). The tracheae were removed and transferred to ice-cold solution containing 136 mM NaCl, 5.36 mM KCl, 0.44 mM KH₂PO₄, 4.16 mM NaHCO₃, 10 mM glucose, 10 mM HEPES, 0.34 mM NaHPO₄·12H₂O (pH 7.1, adjusted with NaOH). The trachealis tissues were isolated and minced and incubated for 22 min at 35°C in the above solution supplemented with 2 mg/mL papain, 1 mg/mL dithioerythritol, and 1 mg/mL bovine serum albumin (BSA). The tissues were transferred to the above solution supplemented with 1 mg/mL collagenase H, 0.15 mg/mL dithioerythritol, and 1 mg/mL BSA and continued to incubate for 8 min. The tissues were washed and gently triturated in the above solution to release single smooth muscle cells. Cells were stored at room temperature and used for experiments.

Recordings of Ion Channel Currents

Currents mediated by LVDCCs and ACh-activated channels were recorded using an EPC-10 patch-clamp amplifier (HEKA, Lambrecht, Germany) at room temperature as previously described with some modifications (Tan et al., 2015; Chepurny et al., 2016). Ba²⁺ was used as the charge carrier of LVDCC currents. The intracellular solution contained (in mM) CsCl 130, EGTA 10, MgCl₂ 4, Mg-ATP 4, HEPES 10, and TEA 10 (pH 7.2, adjusted with CsOH). The extracellular solution contained (in mM) NaCl 105, CsCl 6, BaCl₂ 27.5, glucose 11, HEPES 10, TEA 10, and NA 0.1 (pH 7.4, adjusted with NaOH). The holding potential was −70 mV, and currents were recorded every 10 s using 500 ms steps from -70 to 40 mV in 10 mV increments. For ACh-activated current measurements, the intracellular solution contained (in mM) CsCl 18, cesium acetate 108, MgCl₂ 1.2, HEPES 10, EGTA 3, and CaCl₂ 1 (pH 7.2, adjusted with Tris-HCl). The free Ca²⁺ concentration was approximately 70 nM as calculated using the WEBMAXC standard program. The extracellular solution was PSS without K⁺ containing 10 μM nifedipine, 100 μM NA, and 10 mM TEA to block Ca²⁺, Cl⁻, and K⁺ channel-mediated currents, respectively. Cells were patched in the classical whole-cell configuration and held at −50 mV. Currents were measured using a 500 ms ramp from −80 to 60 mV, and the values at −70 mV were used to represent the current.

Measurement of Intracellular Ca²⁺

Tracheal smooth muscle cells were loaded with 2.5 fluo-4 AM and the intracellular Ca²⁺ was measured and analyzed as previously described using an LSM 700 laser scanning confocal microscope.

1http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm
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**FIGURE 3** | The effect of EESC is reversible. (A) ACh induced similar contractions in TRs after washout of EESC. (B) ACh-caused contractions were similar in TRs treated with EESC for 24 h and controls. NS: \( P > 0.05 \). These data suggest that EESC-induced relaxation is not due to EESC-induced cytotoxicity and inhibition of the contraction-related protein expression.

(Carl Zeiss, Jena, Germany) (Zhang et al., 2014). The background value was subtracted from the fluorescent intensity of cells. The difference values represent the levels of intracellular Ca\(^{2+}\).

**Statistical Analysis**

The results are expressed as the means ± standard error of the mean (SEM). Student’s \( t \)-tests between two groups were performed, and statistical significance was established as \( P < 0.05 \).

**RESULTS**

**EESC Inhibits ASM Contraction**

We first tested whether EESC affects the contraction of tracheal smooth muscle. As shown in Figure 1A, a mouse tracheal ring (TR) contracted after application of 100 \( \mu \)M ACh, and this contraction was then decreased by EESC. The dose-inhibition of EESC was shown, and the IC\(_{50}\) value and maximal inhibition were calculated as 0.51 ± 0.05 mg/mL and 93.93 ± 1.66% (\( n = 8 \)), respectively. In addition, EESC inhibited the 80 mM K\(^{+}\)-induced contraction (Figure 1B). The IC\(_{50}\) was 0.37 ± 0.04 mg/mL, and the maximal inhibition was 104.24 ± 3.12% (\( n = 8 \)). These data indicate that EESC almost completely blocks ACh- and high K\(^{+}\)-induced ASM contraction.

We then assessed whether the EESC-induced relaxation was affected by epithelia. The following experiments were performed in epithelia-denuded TRs (Figures 1C, D). The IC\(_{50}\) value and maximal inhibition were 0.52 ± 0.04 mg/mL and 104.0 ± 8.7% (\( n = 4 \)), and 0.36 ± 0.01 mg/mL and 102.3 ± 1.1% (\( n = 4 \)) for the precontraction induced by ACh and high K\(^{+}\), respectively. These results demonstrate that EESC-evoked relaxation is not mediated...
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FIGURE 4 | EESC via inhibiting ion channel-mediated Ca$^{2+}$ influx inhibits high K$^+$-induced contraction. (A) High K$^+$ (80 mM) did not trigger contraction in a TR in Ca$^{2+}$-free conditions (0 Ca$^{2+}$ and 0.5 mM EGTA). After the addition of 2 mM Ca$^{2+}$, contraction occurred, which was blocked by EESC. (B) After incubation with EESC, the 2 mM Ca$^{2+}$-induced contraction scarcely occurred. Each of the experiments was performed in 8 TRs. These results indicate that EESC-induced relaxation is due to its inhibition of LVDCC-mediated Ca$^{2+}$ influx.

by epithelia. Thereby, the following experiments were conducted in epithelia-intact TRs.

We next studied whether EESC inhibits contraction in bronchial ASM. As shown in Figure 2A, the lumen area of the airway in a mouse lung slice was observed and was decreased by ACh, and this effect was markedly reversed following application of EESC. However, the reversion was not observed in controls (i.e., in the absence of EESC, data not shown). The summary of the results is shown in Figure 2B. These data indicate that EESC inhibits bronchial ASM contraction.

EESC-Induced Relaxation Is Due to Inhibition of Ca$^{2+}$-Permeable Ion Channels

To investigate the underlying mechanism of EESC-induced inhibition on precontraction, we first did two experiments as shown in Figure 3. EESC-induced relaxation is reversible (Figure 3A) and the incubation of 1.78 mg/mL EESC for 24 h did not affect ACh-induced contraction in TRs compared to controls (Figure 3B). These data suggest that EESC-induced relaxation was not due to the cytotoxicity or inhibition on contractile molecules.

High K$^+$ induces activation of LVDCCs, which mediate Ca$^{2+}$ influx and trigger contraction. Thus, we studied whether the EESC-induced relaxation is due to inactivation of LVDCCs. As shown in Figure 4A, 80 mM K$^+$ was used to open LVDCCs inducing contraction, however, 80 mM K$^+$ did not induce

FIGURE 5 | EESC via inhibiting ion channel-mediated Ca$^{2+}$ influx inhibits ACh-induced contraction. (A) A TR was contracted by ACh, and the contraction was inhibited by nifedipine and then blocked by EESC. (B) The contraction was reduced by nifedipine and then blocked by YM-58483. (C) In the presence of nifedipine, ACh induced a transient contraction in Ca$^{2+}$-free conditions (0 Ca$^{2+}$ and 0.5 mM EGTA). After the addition of 2 mM Ca$^{2+}$, a sustained contraction occurred, which was blocked by EESC. (D) These contractions were markedly inhibited in an EESC-incubated TR. Each of the above experiments was conducted in 6–8 TRs, and the same results were observed. These results demonstrate that EESC-induced relaxation is due to the inhibition of LVDCCs and other pathways.
These data suggest that other pathways would include Ca\(^{2+}\) presence of nifedipine and Ca\(^{2+}\) involved in the EESC-induced inhibition of the ACh-induced abolishment of contraction induced by 2 mM Ca\(^{2+}\) EESC. Furthermore, incubation with EESC almost completely blocked the contraction of ASM induced by ACh and high K\(^{+}\) in mouse TRs (Figure 7B), in mouse bronchi by ACh (Figure 7C). These data illustrated that EESC-induced relaxation can be achieved by aurauto-obtusin.

Aurantio-obtusin Is Included in EESC and Results in Relaxation

To determine which compound is responsible for the EESC-induced relaxation, we separated EESC compounds. Semen cassiae has been reported to contain numerous compounds, one of which is aurantio-obtusin (Dong et al., 2017; Kwon et al., 2018). As shown in Figure 7A, one peak of EESC (IR = 25.540) overlapped with that of the standard for aurantio-obtusin (IR = 25.500). These data indicate that aurantio-obtusin is present in EESC. We found that the standard aurantio-obtusin blocked the contraction of ASM induced by ACh and high K\(^{+}\) in mouse TRs (Figure 7B), in mouse bronchi by ACh (Figure 7C). These data illustrated that EESC-induced relaxation can be achieved by aurantio-obtusin.

To further confirm this, aurantio-obtusin was removed from EESC and the remaining components failed to induce relaxation compared to the controls (Figure 8).

Aurantio-obtusin Inhibits Ca\(^{2+}\) Permeant Ion Channels

To study the underlying mechanism of aurantio-obtusin-induced relaxation, we studied whether aurantio-obtusin inhibits LVDCCs and ACh-activated ion channels. LVDCC-mediated currents were measured and which were inhibited by nifedipine and aurantio-obtusin (Figure 9A), indicating that aurantio-obtusin inhibits LVDCCs. Moreover, aurantio-obtusin blocked ACh-induced inward currents from −10.6 ± 2.2 pA to −0.7 ± 0.2 pA (n = 6, P < 0.0005), by YM-58483 from −10.1 ± 2.4 pA to −0.8 ± 0.3 pA (n = 6, P < 0.05), and inhibited by Pyr3 from −11.2 ± 0.3 pA to −4.7 ± 2.6 pA (n = 6, P < 0.05) and further blocked by EESC (Figure 9B). These results indicate that aurantio-obtusin inhibits ACh-activated SOCs and TRPC3/STIM/Orai channels.

To further confirm the above conclusion, we observed the effect of aurantio-obtusin on intracellular Ca\(^{2+}\). The result showed that ACh-induced sustained increases of intracellular Ca\(^{2+}\) were inhibited by aurantio-obtusin (Figure 10), suggesting that the decreases of Ca\(^{2+}\) were due to inhibition of above Ca\(^{2+}\) permeant ion channels by aurantio-obtusin.

EESC Inhibits Human Bronchial ASM Contraction

We finally observed whether EESC and aurantio-obtusin has a similar inhibitory effect on human ASM. As shown in Figure 11, human bronchial ASMs were contracted by ACh, and this contraction was inhibited by EESC and aurantio-obtusin (n = 3 samples from 3 subjects for each experiment).

DISCUSSION

In this study, we found that the ingredients (i.e., EESC and aurantio-obtusin) of Semen cassiae reversibly reduced both ACh- and high K\(^{+}\)-induced preconstrictions in mouse TRs and lung slices. The ACh-induced precontraction was inhibited by the blocker of LVDCCs, SOCs and TRPC3/STIM/Orai.
Aurantio-obtusin blocks ASM contraction. (A) Separation of EESC components and the standard aurantio-obtusin were analyzed with HPLC. One peak of EESC overlapped with that of the standard aurantio-obtusin. These experiments were conducted twice, and the same results were acquired.
(B,C) Aurantio-obtusin blocked the contraction of ASM from TRs induced by ACh and high K⁺, and from lung slices provoked by ACh. These results indicate that aurantio-obtusin, which is one component of EESC, inhibits ASM contraction.
The purpose of this study was to screen for active herbal constituents that inhibit ASM contraction. We found that *Semen cassiae* contains such ingredients. Its extract, EESC, potently inhibited ACh- and high K+ -induced precontraction. When *aurantio-obtusin* was removed from EESC using semi-preparative HPLC, EESC failed to induce relaxation. All these results together indicate that EESC-induced relaxant effects resulted from *aurantio-obtusin*.

The existence of these channels is further confirmed by the existence of nifedipine (Figure 6), thus suggesting that TRPC3 and/or STIM/Orai channels contributed to the contraction. The results showed that Pyr3 partially inhibited the contraction in the presence of nifedipine (Figure 5B). This YM-58483-sensitive Ca2+ influx pathway can also be blocked by EESC based on the Ca2+ restoration experiments (Figures 5C,D). Moreover, this inhibition is also achieved by *aurantio-obtusin*, because it blocked ACh-induced contractions (Figures 7B,C) and SOC-mediated currents (Figure 9B).

It has been reported that Pyr3 sensitive channels play a role in ACh-induced ASM contraction (Fleischmann et al., 1997; Wang et al., 1997; Zhang et al., 2014). Therefore, we observed the effect of Pyr3, a blocker of TRPC3 and STIM/Orai channels, on ACh-induced contraction. The results showed that Pyr3 partially inhibited the contraction in the presence of nifedipine (Figure 6), thus suggesting that TRPC3 and/or STIM/Orai channels contributed to the contraction. The existence of these channels is further confirmed by that Pyr3 inhibited ACh-induced NSCC-mediated currents (Figure 9B).

It has been reported that *Semen cassiae* contains > 70 chemical compounds including *aurantio-obtusin*. Its aqueous and ethanol extracts have hypotensive effects via inhibiting nervous reflex and iNOS/NO-related receptor-controlled calcium channel-mediated Ca2+ influx (Dong et al., 2017). The ethanol extracts include *aurantio-obtusin*, which is one component of EESC. *Semen cassiae* contains such ingredients. Its extract, EESC, potently inhibited ACh- and high K+ -induced precontraction. When *aurantio-obtusin* was removed from EESC using semi-preparative HPLC, EESC failed to induce relaxation. All these results together indicate that EESC-induced relaxant effects resulted from *aurantio-obtusin*.

What is the mechanism underlying EESC-induced relaxation? Firstly, this relaxation results neither from the cytotoxicity of EESC, because its inhibition is reversible (Figure 3A) nor from decreased expression of contractile-related molecules, since we did not observe decrease of ACh-induced contraction in TRs treated with EESC for 24 h (Figure 3B).

High K+ is known to induce activation of LVDCCs, which then mediate Ca2+ influx, subsequently triggering contraction. Our results show that this pathway was blocked by EESC and then resulted in ASM relaxation (Figure 4). Moreover, this effect is achieved by *aurantio-obtusin*, which is one component of EESC (Figure 7A) and blocked LVDCC-mediated contraction (Figure 7B) and currents (Figure 9A).

LVDCCs also involve in ACh-induced precontraction, based on that a selective blocker of LVDCCs, nifedipine, inhibited the contractions (Figure 5A), consistently with previous results (Zhang et al., 2013, 2014). Moreover, these channels are inhibited by *aurantio-obtusin* (Figure 9A). Therefore, EESC-mediated relaxation is partly due to its inhibition of LVDCCs. In addition to LVDCCs, other pathways also involve in EESC-mediated relaxant effect, because EESC further block the contraction in the presence of nifedipine (Figure 5A).

To define the other pathways, we assessed the effect of the selective blocker of SOCs YM-58483 on ACh-induced contraction, since that ACh induces a depletion of Ca2+ store, which will then activate SOCs mediating Ca2+ influx. The results show that SOC-mediated Ca2+ influx plays an important role, because YM-58483 almost blocked the nifedipine-insensitive contraction (Figure 5B). This YM-58483-sensitive Ca2+ influx pathway can also be blocked by EESC based on the Ca2+ restoration experiments (Figures 5C,D). Moreover, this inhibition is also achieved by *aurantio-obtusin*, because it blocked ACh-induced contractions (Figures 7B,C) and SOC-mediated currents (Figure 9B).
Figure 9 | Aurantio-obtusin inhibits ion channel-mediated currents in single tracheal smooth muscle cells. (A) LVDCC-mediated currents were recorded using the voltage steps (top). The representative traces show that the currents are blocked by nifedipine and aurantio-obtusin (middle). The current-voltage curves were plotted (bottom), showing that the currents were completely blocked by nifedipine and markedly inhibited by aurantio-obtusin. *P < 0.05; **P < 0.0001. (B) ACh-induced currents were recorded using a ramp (top). The non-selective cation channel (NSCC)-mediated currents are isolated in the presence of three blockers. The currents at -70 mV were used to construct time-current traces, showing that aurantio-obtusin and YM-58483 completely and Pyr3 greatly inhibited the currents. These results suggest that aurantio-obtusin inhibits nifedipine-, YM-58483- and Pyr3-sensitive ion channels.

described inhibition of Ca\textsuperscript{2+} influx and relaxation (Dong et al., 2017). This link will be supported by that *aurantio-obtusin* induced relaxation in rat mesenteric arteries, but not pulmonary arteries, through endothelial intracellular PI3K/Akt/eNOS/NO signaling pathway (Li et al., 2015), although this mechanism is different from those above described (Dong et al., 2017).

We further showed that *aurantio-obtusin* induces ASM relaxation via inhibiting increased intracellular \textsuperscript{2+}Ca by blocking ACh-activated \textsuperscript{2+}Ca-permeant ion channels, as *aurantio-obtusin* significantly decreased ACh-mediated increasing of intracellular \textsuperscript{2+}Ca (Figure 10), the result was consistent with the previous observation (Dong et al., 2017). \textsuperscript{2+}Ca is a critical modulatory factor for ASM tension and airway...
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**FIGURE 10** | Aurantio-obtusin inhibits ACh-induced Ca\(^{2+}\) increases in single tracheal smooth muscle cells. (A) ACh induced a transient and a sustained increase of intracellular Ca\(^{2+}\). The latter was inhibited following the addition of aurantio-obtusin. The values at the sites indicated by a and b were obtained. (B) Summary results from 26 cells. \(^{***}P < 0.001\). These results indicate that aurantio-obtusin inhibits increased Ca\(^{2+}\).

**FIGURE 11** | EESC and Aurantio-obtusin inhibits ACh-induced Ca\(^{2+}\) increases in human ASM. (A,B) ACh induced a sustained contraction and was inhibited by EESC and aurantio-obtusin, respectively. These results suggest that both have the similar inhibitory action in human ASM.

Moreover, both had the similar inhibitory action on human ASM contraction (**Figure 11**). These results suggest that both can be used to develop potential drugs for asthma and COPD.

In summary, our study demonstrates that EESC-contained *aurantio-obtusin* inhibits ASM contraction through inhibiting Ca\(^{2+}\)-permeable ion channels. These findings suggest that *aurantio-obtusin* may be used to develop novel antiasthmatic drugs.

**AUTHOR CONTRIBUTIONS**

Y-SS, L-QM, and Q-HL designed the experiments. Y-SS, L-QM, Q-HL, B-BL, W-IJ, J-YQ, M-YL, XL, QW, HX, D-AZ, YYC, X-XZ, LC, JS, Y-BP, PZ, LX, M-FY, WC, XN, CS, ShuC, ShaC, GQ, JD, and JC performed the experiments, analyzed the data. Y-SS, L-QM, and Q-HL wrote the paper.

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