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Erythromycin relaxes BALB/c mouse airway smooth muscle

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ABSTRACT

Aims: Bitter taste receptor (TAS2R) agonists have bronchodilatory potentials. Erythromycin is a ligand of TAS2R10, but its relaxant profile is unknown. This study was performed to understand the relaxant effects of erythromycin and its potential mechanism.

Main methods: Airway resistance was tested by the whole body plethysmography in the ovalbumin-aluminum hydroxide induced asthma model mice. Tracheal ring segment myography was used to investigate the isometric tension of the smooth muscle. The cyclic adenosine monophosphate (cAMP) concentration was measured by enzyme immunoassay kit. Changes in the calcium influx in airway smooth muscle cells (ASMCs) were surveyed using a real-time confocal microscopy.

Key findings: Erythromycin significantly relieved airway hyperreactivity in asthma model mice. Erythromycin relaxed mouse tracheal segments precontracted with carbachol, KCl, 5-hydroxytryptamine and U46619, and further dilated the tracheal rings relaxed by isoprenaline or atropine. Epithelium removal, indomethacin or NS-398 partially reduced the relaxation. U73122, 2-APB, iberiotoxin or ouabain did not change the concentration-relaxation curves of erythromycin on tracheal segments. Erythromycin didn't elevate cAMP level. $CaCl_2$-induced contraction in the K^+-rich solution was impaired by erythromycin in the Ca^{2+}-free solution. The intercellular Ca^{2+} level in the ASMCs was decreased by erythromycin, which was partly inhibited by Bay K8644 but not gallein.

Significance: Erythromycin had marked bronchodilatory effect. The relaxation might be related to the L-type voltage-dependent calcium channel, but not the gustducin-associated βγ/phospholipase-Cβ/inositol 1,4,5-triphosphate receptor/large conductance Ca^{2+}-activated K^+ channel pathway or a cAMP-dependent way.

1. Introduction

Macrolides are commonly prescribed to treat infections associated with *Streptococcus pneumoniae*, *Mycoplasma pneumoniae* or *Chlamydophila pneumoniae*. Macrolides also showed distinct anti-inflammatory properties in asthma, chronic obstructive pulmonary diseases and other respiratory inflammatory diseases [1,2]. In this study, we tried to interpret a new pharmacological effect of erythromycin which was certified to be a ligand of bitter taste receptor (TAS2R) subtype 10 [3].

TAS2R has been reported to be widely expressed in extra-oral organs [4]. Recently it has been detected in the lung and 17 TAS2R subtypes are found in human airways [5,6]. TAS2R10, 14, 31 are expressed at the highest levels [7]. Many bitter chemicals are ligands for TAS2R and activate different subtypes [8]. Chloroquine mainly binds to TAS2R1, 10 and 39, whereas quinine activates 9 subtypes, including TAS2R4, 7, 10 and 14 [3,9]. TAS2R agonists exert a relaxant effect on the airway, and different bitter tastants display distinct patterns of relaxation. Colchicine, an activator of TAS2R4 and 46, induces a maximal relaxation of 18.3%, but the largest bronchodilatory effects of chloroquine and quinine are 94.2% and 89.5%, respectively [10]. Denatonium, which agonizes 7 TAS2R subtypes, selectively inhibits contractions induced by carbachol (CCh), but chloroquine inhibits all the contractions evoked by CCh, the thromboxane receptor agonist U46619, leukotriene D4 and histamine [11].

Therefore, the bronchodilatory effects of TAS2R ligands have been confirmed, thus providing new insights into bronchodilators other than traditional β_2 adrenoceptor agonists, muscarinic receptor antagonists and theophylline [10]. But the relaxant efficacy varies from different bitter tastants. Erythromycin, a well-known bitter tasting antibiotic,

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exclusively agonizes TAS2R10, one of the most abundant subtypes [3,7]. As the relaxant profile of erythromycin has not yet been reported, we investigated the dilatory effects of erythromycin on mouse airways and tried to explore its potential mechanism.

2. Materials and methods

2.1. Regents and animals

CCh, methacholine (MCh), U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α), 5-HT (5-hydroxytryptamine), indomethacin, L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride), ouabain, TEA (tetraethylammonium chloride), glibenclamide, thapsigargin, $BaCl_2$, 4-AP (4-aminopyridine), erythromycin, chloroquine and quinine were purchased from Sigma. The cyclic adenosine monophosphate (cAMP) enzyme immunoassay kit, SC-560 (5-(4-Chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole), NS-398 (N-[2-(Cyclohexyloxy)-4-nitrophenyl]methanesulfonamide) and SLIGRL (L-seryl-L-leucyl-L-isoleucyglycyl-L-arginyl-L-leucinamide) were purchased from Cayman Chemical Company. The salbutamol sulfate solution for inhalation was purchased from Glaxo Smith Kline. Verapamil hydrochloride injection and isoprenaline hydrochloride injection were obtained from Shanghai Hefeng Pharmaceutical Co., Ltd. Erythromycin lactobionate for injection was bought from Dalian Meiluo Pharmaceutical Co., Ltd. Bay K8644 (1,4-Dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)pyridine-3-carboxylic acid methyl ester) and U73122 (1H-Pyrrole-2,5-dione, 1-[6-[[(17b)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]) were purchased from Medchem Express. Iberiotoxin and gallein were purchased from EMD Millipore Corporation. 2-APB (2-aminoethoxydiphenyl borate) was obtained from StressMarq Biosciences. Fluo-3 AM was bought from Biotium.

The Animal Center of Basic Medical Sciences School of Xi'an Jiaotong University provided the eight-week-old female BALB/c mice, which were bred and caged in a well-controlled environment, with temperature and humidity as 20–22 °C and 60%–80%, respectively. All mice were provided with a normal diet and could reach food and water freely. The experiment protocol was approved by Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University (document number: 2016060, approval date: Mar 3, 2016).

2.2. Asthma model and AHR measurements

The BALB/c mice were divided into control (10 mice) and model group (40 mice) randomly. The model mice were immunized by intraperitoneal injection of ovalbumin-aluminum hydroxide (100 μg: 5000 μg) 100μL per mouse. Two weeks later, 40μL of ovalbumin-PBS (containing 40 μg ovalbumin) was instilled intranasally in the sensitized group for 7 days. The control mice received the same amount of PBS.

AHR was tested by the DSI Buxco whole body plethysmography and Penh value was used as the indicator of airway resistance. After the last challenge, the control mice and 10 model mice received a series concentration of MCh (1 mg/mL–64 mg/mL) to record the Penh-concentration curves. The other 30 model mice were divided into 3 groups (PBS, salbutamol or erythromycin), each mouse was administrated MCh (16 mg/mL) to increase baseline airway resistance, then PBS, salbutamol sulfate solution for inhalation or erythromycin lactobionate for injection was aerosolized and inhaled by the mice to relieve the bronchospasm.

2.3. Tracheal ring segment myography

The untreated BALB/c mice were intraperitoneally injected with sodium pentobarbital (150 mg/kg) and the whole tracheae were gently removed. Then the trachea was cut into 3–4 rings, with each having two cartilage rings. The ring segments were submerged in baths, including 5 mL of 3-morpholinopropanesulfonic acid (MOPS) solution (in mM: NaCl 140, MOPS 2.0, $Na_2HPO_4{\cdot}12H_2O$ 1.2, EDTA 0.02, KCl 4.7, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2$ 1.6, and glucose 5.6, adjusted the pH to 7.4), which were continuously treated with O_2 [12]. Each tracheal segment was mounted on two metal silk threads, one thread was connected to a force-displacement transducer which was attached to a digital converter unit (AD Instruments, Hastings, UK), and the other thread was associated with a displacement device. Thus, adjustment of the distance between the two threads could be allowed and isometric tension of tracheal rings was continuously recorded. A pre-tension of 0.8 mN was applied to each ring for 1 h before experiments [13].

The segments were contracted with 60 mM KCl for evaluating contractile activity. The plateau tension was obtained with CCh, 5-HT or U46619 and then erythromycin, chloroquine, quinine, isoprenaline, salbutamol, atropine or ipratropium bromide was cumulatively applied to baths for testing the relaxant effects. Inhibitors as L-NAME, indomethacin, ouabain, TEA, glibenclamide, iberiotoxin, thapsigargin, 2-APB or U73122 was incubated with the tracheal ring segments for 30 min before the contractile agents were added [3].

The mouse tracheal ring segments were immersed in a Ca^{2+}-free solution (same components as the MOPS solution except for the absence of $CaCl_2$ and addition of 0.1 mM EDTA) and incubated with erythromycin, chloroquine or verapamil for 30 min; then, CCh was applied to induce intracellular calcium release. Next, $CaCl_2$ (2 mM) was added to induce contraction attributed to extracellular calcium influx. Mouse tracheal segments were immersed in a K^+-rich solution (no $CaCl_2$, containing 0.1 mM EDTA and 60 mM KCl), and incubated with verapamil, erythromycin or chloroquine for 20 min, and then $CaCl_2$ (10 mM) was added to explore the role of the L-type voltage-dependent calcium channel (L-VDCC) in the relaxant effects of these chemicals [14].

Epithelium was removed from trachea using the approach proposed by Cocks et al [15,16]. Temporarily, the whole trachea was gently flushed a MOPS solution containing 0.1% Triton X-100 into *in situ* before the dissection. The segment rings were contracted with 60 mM KCl, then washed three times and CCh (0.1 μM) was added, then responsiveness to the proteinase activated receptors 2 agonist SLIGRL (10 μM) was examined to test functional effectiveness of removing epithelium. Thereafter, all the segments were washed and KCl (60 mM) was used again to check the airway smooth muscle integrity.

2.4. Airway smooth muscle cells (ASMCs) culture, cAMP and intercellular Ca^{2+} measurements

The untreated BALB/c mice were sacrificed and then the tracheae were isolated and transferred to an ice-cold phosphate buffer solution (PBS) containing 1% penicillin and streptomycin. The epithelium was scraped softly, and then the tracheal tissues were cut into 1 mm^3 pieces and incubated for 30 min with 0.2% collagenase I and a following incubation with 0.2% collagenase I and trypsin for 30 min. The partially digested tissues were then washed 3 times and transferred to DMEM (containing 20% FBS) to yield single ASMCs.

The ASMCs were suspended (5×10^4 cells/well) in a 96-well plate, and treated with erythromycin (1 mM), salbutamol (0.1 μM) or chloroquine (0.1 mM) for 60 min. Then the cAMP concentration (pmol/mL) was measured according to the manufacturer's protocol of enzyme immunoassay kit [17,18].

Intracellular Ca^{2+} levels in ASMCs were measured and fluo-3/AM was used as calcium indicator. After loading with fluo-3/AM (5 μM), the cells were incubated with or without gallein or Bay K8644, then KCl (60 mM) and subsequent erythromycin were added. The fluorescence frames were continuously acquired every 1.107 s through an Olympus Fluoview-500 confocal system (Olympus, Tokyo, Japan). The change rate of fluorescence intensity (F/F_0%) was calculated from individual image using FV-ASW software (version 1.7, Olympus, Tokyo, Japan). [19]

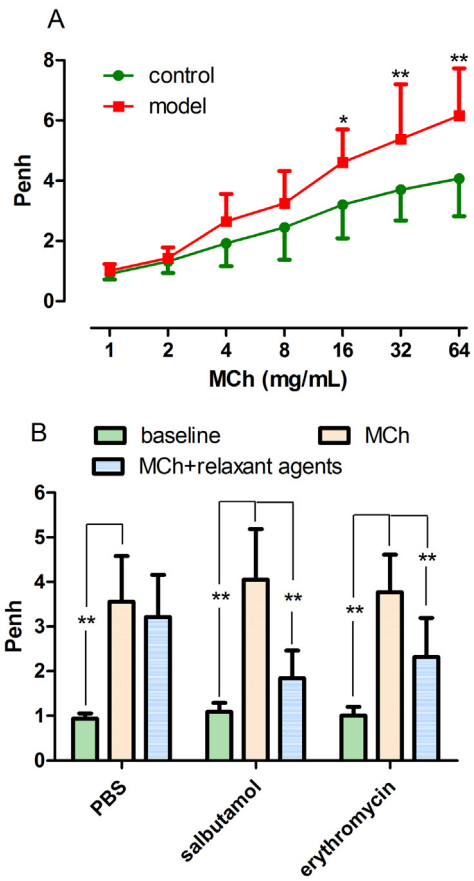

Fig. 1. Erythromycin eased AHR in asthma model mice. BALB/c model mice was duplicated by ovalbumin-aluminum hydroxide and airway resistance was tested by the whole body plethysmography. After the last challenge, the control mice and model mice received a series concentration of MCh (1 mg/mL–64 mg/mL) to record the Penh-concentration curves (A). Model mice were administrated with MCh (16 mg/mL) to increase baseline airway resistance, then PBS, salbutamol (5 μg) or erythromycin (450 μg) was aerosolized and inhaled, then the Penh value was recorded (B). Results are presented as means ± SD, n = 10.

2.5. Statistical analysis

Results are expressed as means ± SD and n was related to the number of animals. The maximal dilatory response (R_{max}) was expressed as a percentage of the constrictor (KCl, CCh, 5-HT or U46619)-induced precontraction. pD_2 was the negative logarithm of the agonist concentration which elicited the half maximal response. All data was analyzed by GraphPad Prism 5.01 software. One-way or two-way analysis of variance (ANOVA) with Dunnett's post-test was adopted for the comparison between groups. $P < 0.05$ was thought to be statistically significant.

3. Results

3.1. Erythromycin alleviated AHR in asthma model mice

The OVA-Alum induced asthma model mice displayed significant hyperreactivity compared to the control mice (Fig. 1A). When inhaled salbutamol (5 μg) or erythromycin (450 μg), the pulmonary function of the mice was improved, as the Penh value had apparent reduction in the salbutamol group (4.05 ± 1.13 *vs* 1.84 ± 0.62, $P < 0.05$) and erythromycin group (3.77 ± 0.84 *vs* 2.32 ± 0.87, $P < 0.05$) (Fig. 1B).

3.2. Erythromycin relaxed ex vivo tracheal segments precontracted by KCl, CCh, 5-HT or U46619

Erythromycin relaxed tracheal segments precontracted with KCl, CCh, U46619 and 5-HT (Fig. 2). Chloroquine, quinine, isoprenaline, salbutamol, atropine, and ipratropium bromide also relaxed the tracheal rings. The R_{max} values for erythromycin and chloroquine were similar or higher than the ones for isoproterenol and salbutamol, but the pD_2 values were much lower than the β_2 adrenoceptor agonists and the muscarinic receptor antagonists (Table 1). The R_{max} value was 71% ± 13% and the pD_2 was 4.18 ± 0.08 in U46619 precontracted tracheal rings. In 5-HT precontracted segments, The R_{max} and the pD_2 value were 68% ± 18% and 4.27 ± 0.10, respectively.

3.3. Erythromycin had additional relaxations on tracheal rings dilated by β adrenoceptor agonists or muscarinic receptors antagonists

When isoproterenol (or atropine) produced its maximal relaxation on the tracheal segments, the subsequent addition of salbutamol (or ipratropium bromide) did not induce further dilation, indicating that the β adrenoceptors or muscarinic receptors were full occupied. But erythromycin further relaxed the isoproterenol- or atropine-relaxed airway to reach a maximal relaxation of nearly 100% (Fig. 3A–C).

3.4. The epithelium was involved in the relaxant effect of erythromycin

As shown in Fig. 4, the relaxation of erythromycin in the epithelium denuded group was lower than that of the epithelium intact group in the concentration of 10^{-3} M (97% ± 10% *vs* 81% ± 13%, $P < 0.01$). The pD_2 values in relaxant curves of erythromycin were 3.29 ± 0.10 and 3.25 ± 0.14 in epithelium intact and denuded tracheal segments. The endothelial nitric oxide synthase inhibitor L-NAME did not change concentration-relaxation curves of erythromycin. The non-selective cyclooxygenase (COX) inhibitor, indomethacin decreased the R_{max} (96% ± 12% *vs* 80% ± 8%, $P < 0.01$) but not the pD_2 values (3.24 ± 0.10 *vs* 3.26 ± 0.10). What's more, selective COX-2 inhibitor NS-398, but not COX-1 inhibitor SC-560, impaired the bronchodilation of erythromycin with the reduction of the R_{max} (98% ± 11% *vs* 84% ± 14%, $P < 0.05$) but not the pD_2 values (3.25 ± 0.11 *vs* 3.28 ± 0.11).

3.5. The relaxant effect of erythromycin did not involve the gustducin-associated βγ ($G_{\beta\gamma}$)/phospholipase-Cβ (PLCβ)/inositol 1,4,5-tri-phosphate receptor (IP3R)/large conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) pathway or cAMP-dependent mechanism

TAS2R agonists were thought to signal through Gβγ activation of PLCβ, generating inositol 1,4,5-tri-phosphate and binding to IP3R that stimulates calcium release and subsequently leads to the activation of the BK_{Ca} which causes membrane hyperpolarization and the marked relaxation of airway smooth muscle [7].

In this study, we tested the effect of Gβγ and its downstream signal molecules in the bronchodilation of erythromycin. When KCl (60 mM) was applicated, the intracellular Ca^{2+} quickly increased and then maintained a plateau. The Ca^{2+} plateau was inhibited following the addition of erythromycin. When the ASMCs were incubated with gallein for 30 min, the reduction of intracellular Ca^{2+} induced by erythromycin was not affected (Fig. 5 A). In the *ex vivo* tracheal segments, PLCβ inhibitor U73122, IP3R antagonist 2-APB, BK_{Ca} inhibitor iberiotoxin or Na^+-K^+-ATPase inhibitor ouabain did not change the relaxant curves of erythromycin (Fig. 5B, C), suggesting that the Gβγ/PLCβ/IP3R/BK_{Ca} pathway was not involved in the effects of erythromycin. Moreover, the non-selective potassium channel inhibitor TEA, the ATP-sensitive potassium channel inhibitor glibenclamide, the inward rectifier potassium channel inhibitor $BaCl_2$ or the voltage-gated potassium channel inhibitor 4-AP did not change the concentration-

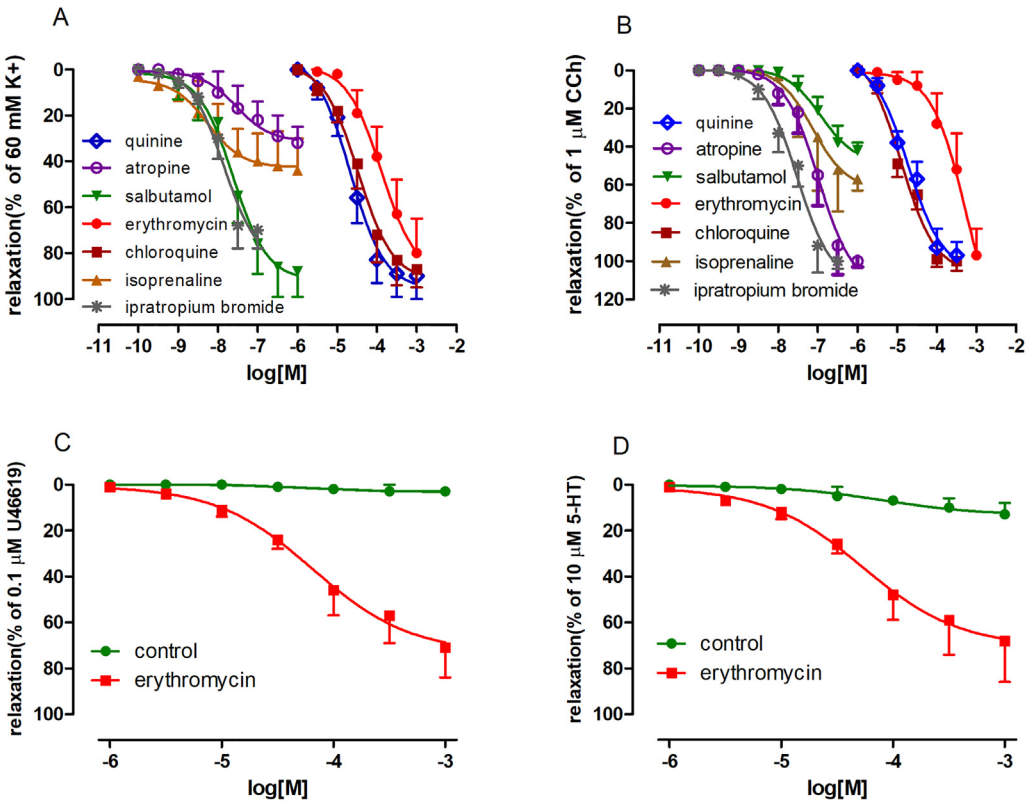

Fig. 2. The relaxant effect of erythromycin on mouse tracheal segments precontracted with KCl, CCh, 5-HT or U46619. Plateau tension was obtained with KCl (60 mM, A), CCh (1 μM, B), U46619 (0.1 μM, C) or 5-HT (10 μM, D) and then bronchodilators were cumulatively administrated to record the relaxant curves. Results are presented as means ± SD, n = 8–16.

Table 1
The maximal relaxation (R_{max}) and the half maximal response (pD_2) of all dilatory agents on the KCl (60 mM)- and CCh (1 μM)-precontracted mouse tracheal segments (means ± SD, n = 8–16).

Chemicals	KCl-precontracted		CCh-precontracted	
	R_{max} (%)	pD_2	R_{max} (%)	pD_2
Erythromycin	80 ± 15	3.89 ± 0.07	97 ± 14	3.26 ± 0.12
Chloroquine	87 ± 8	4.47 ± 0.06	100 ± 5	4.90 ± 0.06
Quinine	90 ± 10	4.67 ± 0.06	97 ± 7	4.72 ± 0.06
Isoprenaline	44 ± 14	8.26 ± 0.19	57 ± 6	7.13 ± 0.12
Salbutamol	88 ± 11	7.62 ± 0.06	42 ± 4	6.92 ± 0.07
Atropine	32 ± 7	7.54 ± 0.13	100 ± 3	6.99 ± 0.06
Ipratropium bromide	70 ± 8	7.85 ± 0.07	100 ± 4	7.52 ± 0.06

response curves of erythromycin (Fig. 6A), implying that the relaxant effect did not depend on these potassium channels. In addition, the cAMP level was not increased by the erythromycin treatment (Fig. 6B), indicating that cAMP production was not required for this process.

3.6. L-VDCC might play a role in the relaxant effect of erythromycin

Bitter tastants have been shown to inhibit L-VDCC to induce bronchodilation in primary ASMCs [20]. L-VDCC dominate KCl-induced contraction, but it is much less important in agonist-induced contraction [21]. In our study, KCl-induced contraction was significantly decreased when the tracheal segments were incubated with erythromycin or chloroquine, whereas verapamil completely abolished this contraction (Fig. 7A). Meanwhile, erythromycin and chloroquine alleviated the CCh-induced contraction (Fig. 7B).

The sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin did not affect the relaxation induced by erythromycin (Fig. 7C). In the Ca^{2+}-free solution, erythromycin, chloroquine, or verapamil did not affect the CCh-induced airway smooth muscle contraction, which was thought to be attributed to intracellular calcium release. However, all of these agents obviously inhibited the subsequent $CaCl_2$-induced contraction, which was caused by extracellular calcium influx (Fig. 7D).

In a K^+-rich solution, verapamil, erythromycin or chloroquine significantly inhibited $CaCl_2$ (10 mM)-induced contraction, which was attributed to the L-VDCC (Fig. 7E). Moreover, the Ca^{2+} levels of ASMCs were decreased by erythromycin, which was partly inhibited by Bay K8644, an L-type voltage-gated calcium channel activator, implying that L-VDCC might be involved in the relaxant effects of erythromycin (Fig. 7F).

4. Discussion

The present study demonstrated that erythromycin had massive dilatory effect on airway smooth muscle; it relieved AHR in asthma model mice and relaxed the tracheal rings precontracted by all the tested constrictors. The relaxation in the *ex vivo* tracheal segments seemed equivalent as the traditional β_2-adrenoceptor agonists or muscarinic antagonists, but the pD_2 values were much lower. What's more, erythromycin had additional relaxations on tracheal rings dilated by isoprenaline or atropine. The further relaxation induced by erythromycin also implied new mechanisms that were different from adrenoreceptor agonists and the muscarinic receptor antagonists were involved. [22]

The mechanism of erythromycin induced relaxation was explored in the present study. Epithelial removal induced small decrease of the R_{max} (still > 80%), suggesting epithelium or potential epithelium-derived factors played a weak role in the relaxant effect. A similar study showed that another macrolide, azithromycin, relaxed rabbit tracheal strips *in vitro* [23]. The authors concluded that the relaxant effect of azithromycin was obvious, the R_{max} in epithelium-denuded groups seemed smaller than the epithelium-intact group. In addition, the non-selective COX inhibitor indomethacin and the COX-2 inhibitor NS-398 impaired the relaxant effects, suggesting that erythromycin may slightly influence the effects of prostaglandins (PGEs) which are prominent mediators of bronchial tone. Similar results were observed in

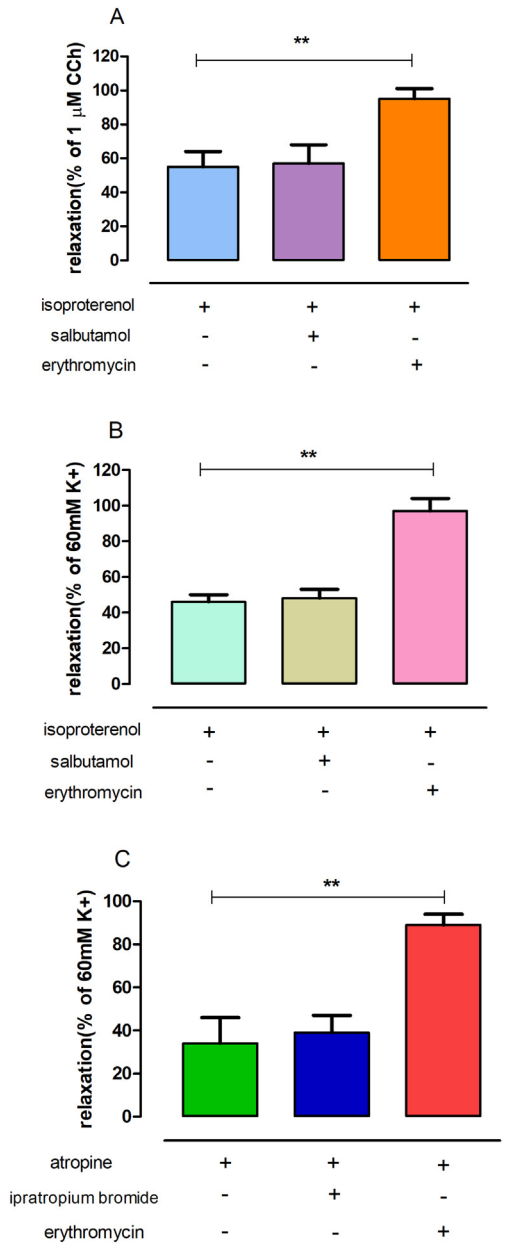

Fig. 3. The extra relaxant effect of erythromycin on tracheal segments in the present of isoproterenol or atropine. Mouse tracheal segments were contracted with CCh (1 μM, A) or KCl (60 mM, B, C), then isoproterenol (1 μM, A, B) or atropine (1 μM, C) was used to produce maximal relaxation; thereafter, salbutamol (1 μM, A, B) or ipratropium bromide (0.1 μM, C) or erythromycin (1 mM, A-C) was applied. Results are presented as means ± SD, n = 8. $^{**}P < 0.01$ compared to the isoproterenol group (A, B) or atropine group (C).

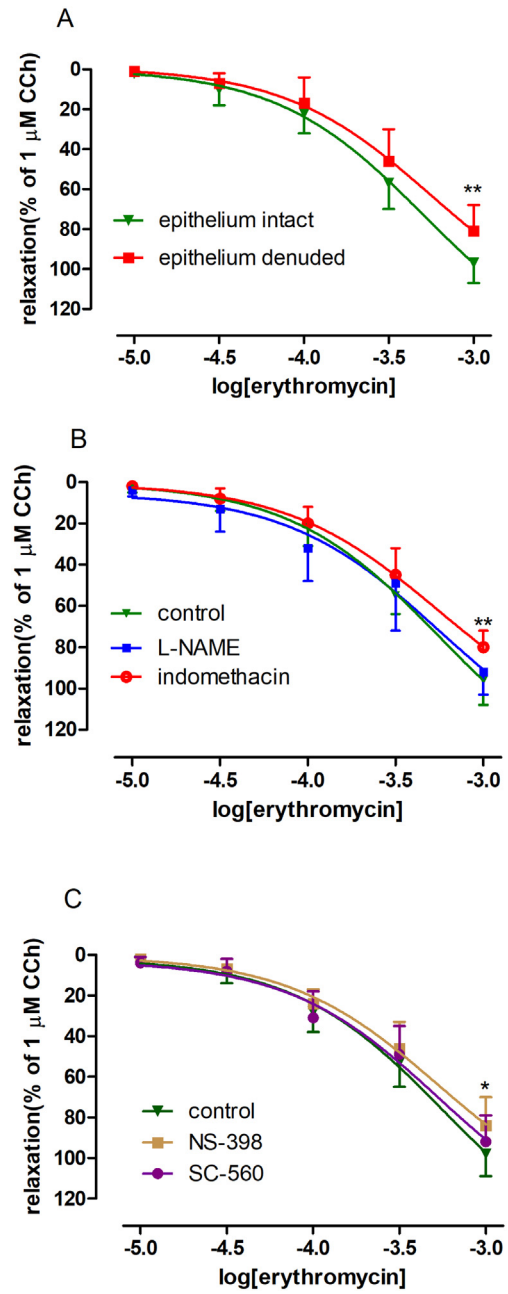

Fig. 4. The relaxant effect of erythromycin on the epithelium-intact or epithelium-denuded mouse tracheal segments (A), L-NAME (3 mM, B) or indomethacin (3 μM, B) incubated mouse tracheal segments and SC-560 (10 μM, C) or NS-398 (10 μM, C) incubated mouse tracheal segments. Results are presented as means ± SD; n = 8–10. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the epithelium intact group (A) or the control group (B, C).

flufenamic acid, a TAS2R14 agonist, inhibited the COX responsible for producing prostaglandins (PGEs) [3]. Phenanthroline, a TAS2R5 agonist, displayed a diminished relaxant effect on epithelium-denuded bronchus [3]. Therefore, epithelium or epithelium-derived factors might play a role in the signaling pathway of some TAS2R agonists and may also be involved in the bronchodilatory effects of erythromycin. However, the effect of epithelium in the relaxation of erythromycin seemed limited.

Airway smooth muscle (ASM) plays a dominant role in bronchial state and the changes of $[Ca^{2+}]_i$ in ASMCs directly leads to bronchial constriction or relaxation [24]. But the mechanism of TAS2R agonists induced relaxation on ASMCs seemed ambiguous. Desphande et al. thought that TAS2R agonists induced a localized $[Ca^{2+}]_i$ response in the Gβγ-PLCβ-IP3R-dependent pathway, followed by BK_{Ca} channel opening and subsequent ASM membrane hyperpolarization [7,25]. In contrast, Zhang et al reported that chloroquine didn't alter localized Ca^{2+} events and spontaneous local Ca^{2+} transients, but inhibited L-VDCC to induce bronchodilation in primary ASMCs [20]. In a study of human bronchi, inhibitors of BK_{Ca} channels and the sarcoplasmic reticulum Ca^{2+}-ATPase did not affect chloroquine- or phenanthroline-induced relaxation, but L-VDCC and the Na^+/K^+ exchanger played a role in the relaxant effects of chloroquine [3], consistent with the findings in ASM cells. However, the authors found that relaxation induced by other TAS2R agonists, dapsone and flufenamic acid, was not affected by ouabain and Bay K8644 at all, suggesting that modulation of

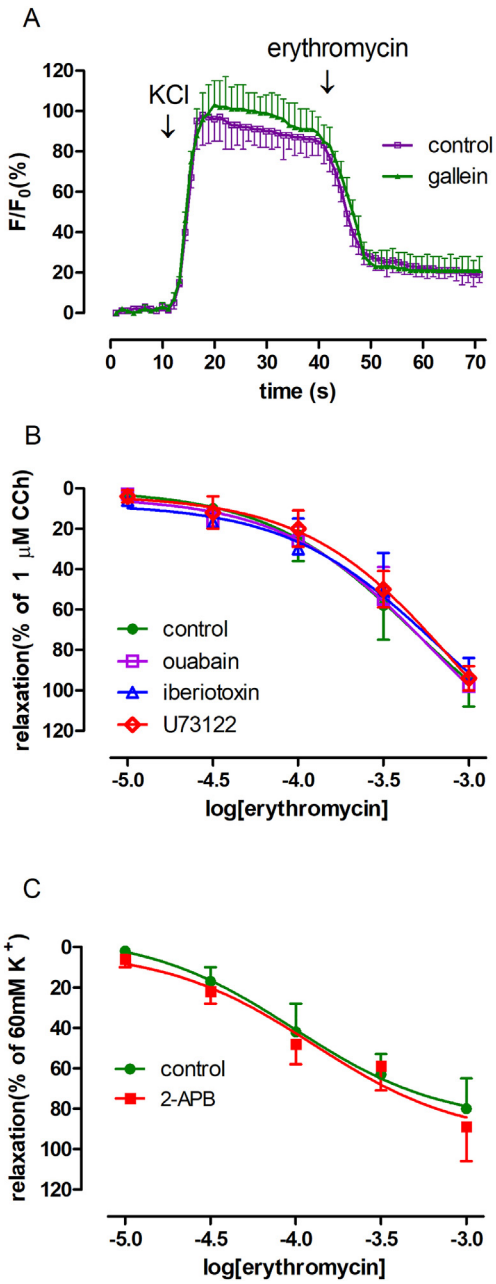

Fig. 5. Gβγ/PLCβ/IP3R/BK_{Ca} pathway was not involved in the relaxant effect of erythromycin. The ASMCs were incubated with gallein(100 μM) and then KCl (60 mM) and subsequent erythromycin were added. F/F0% was measured by Olympus Fluoview-500 confocal system in ASMCs (A).Mouse tracheal segments were incubated with U73122 (1 μM, B), iberiotoxin (0.1 μM, B), ouabain (10 μM, B), 2-APB (50 μM, C) for 30 min; then CCh (1 μM, B) or KCl (60 mM, C) was added and then relaxant curves of erythromycin were recorded. Results are presented as means ± SD; n = 8–12. $^{**}P < 0.01$ compared to the control group.

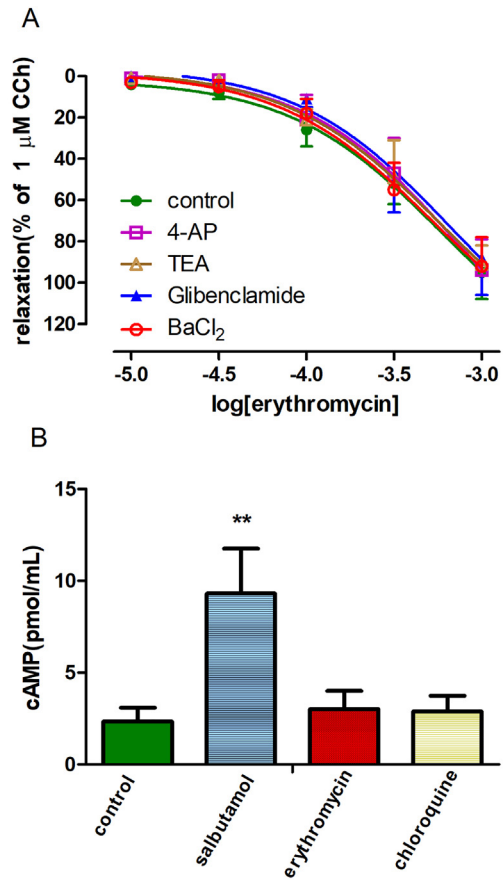

Fig. 6. K^+ channel or cAMP production were not involved in the relaxant effect of erythromycin. Mouse tracheal segments were incubated with the potassium channels blockers, TEA (10 μM), glibenclamide (10 μM), $BaCl_2$ (10 μM) or 4-AP (10 μM) for 30 min, then CCh (1 μM) was added and the relaxant curves of erythromycin were recorded (A). The cAMP level (pmol/mL) was detected in ASMCs stimulated by erythromycin (1 mM), salbutamol (1 μM) or chloroquine (0.1 mM) (B). Results are presented as means ± SD; n = 8. $^{**}P < 0.01$ compared to the control group.

calcium channels was not sufficient to fully elucidate the TAS2R agonists-induced bronchodilation. Others thought that different bitter chemicals may have distinct signaling mechanisms. The effects of denatonium were partially inhibited by BK_{Ca} blockers in guinea pig tracheal segments, but chloroquine-mediated relaxation appeared to be signaling-independent [11]. Our results were consistent with the results reported by Zhang et al., which displayed the role of L-VDCC in the relaxant effects of erythromycin. But Gβγ blockage didn't affect the inhibition of $[Ca^{2+}]_i$ in our present work, which interpreted the bronchodilation induced by erythromycin was mediated in a Gβγ independent pathway. Tan et al. postulated that TAS2R10 agonists reversed agonist-induced constriction by inhibiting Ca^{2+} oscillations and Ca^{2+} sensitivity of ASM cells, and blockade of Gβγ or Gαi signaling failed to attenuate the bronchodilation of chloroquine. Thus, the relaxant effects of TAS2R agonists might be mediated by Gα-gustducin or other G-proteins that interact with TAS2R [26].

There are some limitations in this study. First, the methods mentioned above supplied indirect evidence for the role of L-VDCC. Measurements of ion channel currents of L-VDCC in ASMCs by patch clamp techniques are direct methods to confirm the role of L-VDCC. In further studies, patch clamp techniques should be a key consideration to confirm whether erythromycin relaxes airways *via* L-VDCC. Second, as we know, non-selective cation channels (NSCC) was very important in CCh-induced contraction [27]. Transient receptor potential (TRPC) channels, especially TRPC3 channels are predominantly constitute the activity of NSCCs and mediate extracellular Ca^{2+} influx in ASMCs [28]. Zhang et al also reported chloroquine relaxed mouse tracheal smooth muscle by opening TRPC3 channels [29]. Our results showed erythromycin could completely relax CCh-precontracted airway rings, thus NSCC might be involved in the relaxation. Further studies should be focus on whether NSCC, especially TRPC3, is involved in the relaxation of erythromycin.

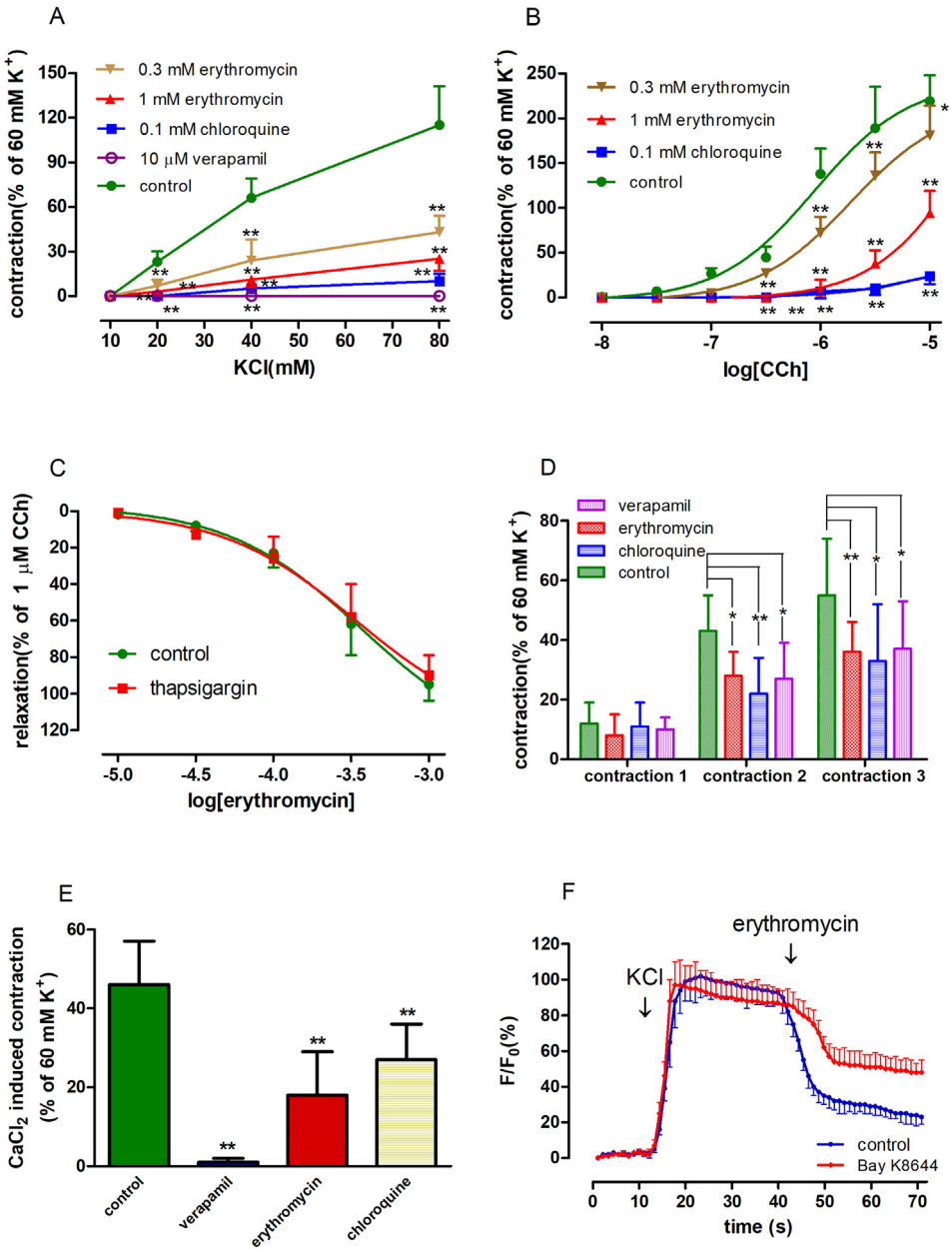

Fig. 7. Erythromycin might relax the tracheal rings *via* L-VDCC dependent pathway. Mouse tracheal rings were incubated with erythromycin (0.3 mM or 1 mM) or chloroquine (0.1 mM) for 30 min, thereafter, KCl (10 mM–80 mM, A) or CCh (1 nM–10 μM, B) was added (in the presence of the tested drugs), and the contraction curves were recorded. Mouse tracheal segments were incubated with thapsigargin (0.1 μM) for 30 min; then, CCh (1 μM) was added and the relaxant curves of erythromycin were recorded (C). The mouse tracheal ring segments were immersed in a Ca^{2+}-free solution and incubated with erythromycin (1 mM), chloroquine (0.1 mM) or verapamil (10 μM) for 30 min. Next, CCh (10 μM) was applied to induce a constrictive plateau (contraction 1). Then, $CaCl_2$ (2 mM) was added and induced a constriction (contraction 2). Contraction 3 was the sum of contraction 1 and contraction 2 (D). Mouse tracheal segments were immersed in a K^+-rich solution, incubated with verapamil (10 μM), erythromycin (1 mM) or chloroquine for 20 min, and then $CaCl_2$ (10 mM) was added to obtain its maximal contraction (E). The ASMCs were incubated with Bay K8644 (1 μM) and then KCl (60 mM) and subsequent erythromycin were added, F/F0% was measured by Olympus Fluoview-500 confocal system (F). Results are presented as means ± SD; n = 8–12. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the control group.

5. Conclusion

Our study showed the bronchodilatory effects of erythromycin on the mouse airway smooth muscle, and epithelium-derived factors and L-VDCC might play a role in the relaxation. These findings expand our recognition about TAS2R agonists and their potential value in the treatment of obstructive airway diseases [10,30].

Conflict of interest

The authors declare that there is no conflict of interest with this study.

Acknowledgments

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References

[1] X. Tong, T. Guo, S. Liu, S. Peng, Z. Yan, X. Yang, et al., Macrolide antibiotics for treatment of asthma in adults: a meta-analysis of 18 randomized controlled clinical studies, Pulm. Pharmacol. Ther. 31 (2015) 99–108.
[2] J. Reiter, N. Demirel, A. Mendy, J. Gasana, E.R. Vieira, A.A. Colin, et al., Macrolides for the long-term management of asthma—a meta-analysis of randomized clinical trials, Allergy 68 (2013) 1040–1049.
[3] S. Grassin-Delyle, C. Abrial, S. Fayad-Kobeissi, M. Brollo, C. Faisy, J.C. Alvarez, et al., The expression and relaxant effect of bitter taste receptors in human bronchi, Respir. Res. 14 (2013) 134.
[4] J.G. Chen, N.N. Ping, D. Liang, M.Y. Li, Y.N. Mi, S. Li, et al., The expression of bitter taste receptors in mesenteric, cerebral and omental arteries, Life Sci. 170 (2017) 16–24.
[5] R. Einstein, H. Jordan, W. Zhou, M. Brenner, E.G. Moses, S.B. Liggett, Alternative splicing of the G protein-coupled receptor superfamily in human airway smooth muscle diversifies the complement of receptors, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 5230–5235.
[6] S.C. Kinnamon, Taste receptor signalling - from tongues to lungs, Acta Physiol (Oxford) 204 (2012) 158–168.
[7] D.A. Deshpande, W.C. Wang, E.L. McIlmoyle, K.S. Robinett, R.M. Schillinger, S.S. An, et al., Bitter taste receptors on airway smooth muscle bronchodilate by localized calcium signaling and reverse obstruction, Nat. Med. 16 (2010) 1299–1304.

[8] T. Kenakin, M. Williams, Defining and characterizing drug/compound function, Biochem. Pharmacol. 87 (2014) 40–63.
[9] M. Ji, X. Su, X. Su, Y. Chen, W. Huang, J. Zhang, et al., Identification of novel compounds for human bitter taste receptors, Chem. Biol. Drug Des. 84 (2014) 63–74.
[10] S. Grassin-Delyle, E. Naline, P. Devillier, Taste receptors in asthma, Curr. Opin. Allergy Clin. Immunol. 15 (2015) 63–69.
[11] V. Pulkkinen, M.L. Manson, J. Safholm, M. Adner, S.E. Dahlen, The bitter taste receptor (TAS2R) agonists denatonium and chloroquine display distinct patterns of relaxation of the guinea pig trachea, Am. J. Phys. Lung Cell. Mol. Phys. 303 (2012) L956–L966.
[12] R. Wang, X. Xiao, Z. Shen, L. Cao, Y. Cao, Airborne fine particulate matter causes murine bronchial hyperreactivity via MAPK pathway-mediated M3 muscarinic receptor upregulation, Environ. Toxicol. 32 (2016) 371–381.
[13] Y. Zhang, Xu CB, L.O. Cardell, Long-term exposure to IL-1beta enhances Toll-IL-1 receptor-mediated inflammatory signaling in murine airway hyperresponsiveness, Eur. Cytokine Netw. 20 (2009) 148–156.
[14] T. Sun, R. Liu, Y.X. Cao, Vasorelaxant and antihypertensive effects of formononetin through endothelium-dependent and -independent mechanisms, Acta Pharmacol. Sin. 32 (2011) 1009–1018.
[15] R.W. Balzary, T.M. Cocks, Lipopolysaccharide induces epithelium- and prostaglandin E(2)-dependent relaxation of mouse isolated trachea through activation of cyclooxygenase (COX)-1 and COX-2, J. Pharmacol. Exp. Ther. 317 (2006) 806–812.
[16] T.M. Cocks, B. Fong, J.M. Chow, G.P. Anderson, A.G. Frauman, R.G. Goldie, et al., A protective role for protease-activated receptors in the airways, Nature 398 (1999) 156–160.
[17] D. Van Ly, M. De Pedro, P. James, L. Morgan, J.L. Black, J.K. Burgess, et al., Inhibition of phosphodiesterase 4 modulates cytokine induction from toll like receptor activated, but not rhinovirus infected, primary human airway smooth muscle, Respir. Res. 14 (2013) 127.
[18] N. Shaikh, M. Johnson, D.A. Hall, K.F. Chung, J.H. Riley, S. Worsley, et al., Intracellular interactions of umeclidinium and vilanterol in human airway smooth muscle, Int. J. Chron. Obstruct. Pulmon. Dis. 12 (2017) 1903–1913.
[19] S. Li, N.N. Ping, L. Cao, Y.N. Mi, Y.X. Cao, H2S induces vasoconstriction of rat cerebral arteries via cAMP/adenylyl cyclase pathway, Toxicol. Appl. Pharmacol. 289 (2015) 389–396.
[20] C.H. Zhang, L.M. Lifshitz, K.F. Uy, M. Ikebe, K.E. Fogarty, R. ZhuGe, The cellular and molecular basis of bitter tastant-induced bronchodilation, PLoS Biol. 11 (2013) e1001501.
[21] Y. Bai, M. Edelmann, M.J. Sanderson, The contribution of inositol 1,4,5-trisphosphate and ryanodine receptors to agonist-induced Ca(2+) signaling of airway smooth muscle cells, Am. J. Phys. Lung Cell. Mol. Phys. 297 (2009) L347–L361.
[22] S.S. An, W.C. Wang, C.J. Koziol-White, K. Ahn, D.Y. Lee, R.C. Kurten, et al., TAS2R activation promotes airway smooth muscle relaxation despite beta(2)-adrenergic receptor tachyphylaxis, Am. J. Phys. Lung Cell. Mol. Phys. 303 (2012) L304–L311.
[23] C. Daenas, A.A. Hatziefthimiou, K.I. Gourgoulianis, P.A. Molyvdas, Azithromycin has a direct relaxant effect on precontracted airway smooth muscle, Eur. J. Pharmacol. 553 (2006) 280–287.
[24] B. Camoretti-Mercado, S.H. Pauer, H.M. Yong, D.C. Smith, D.A. Deshpande, S.S. An, et al., Pleiotropic effects of bitter taste receptors on [Ca2+]i mobilization, hyperpolarization, and relaxation of human airway smooth muscle cells, PLoS One 10 (2015) e0131582.
[25] K.S. Robinett, D.A. Deshpande, M.M. Malone, S.B. Liggett, Agonist-promoted homologous desensitization of human airway smooth muscle bitter taste receptors, Am. J. Respir. Cell Mol. Biol. 45 (2011) 1069–1074.
[26] X. Tan, M.J. Sanderson, Bitter tasting compounds dilate airways by inhibiting airway smooth muscle calcium oscillations and calcium sensitivity, Br. J. Pharmacol. 171 (2014) 646–662.
[27] J.M. Dai, K.H. Kuo, J.M. Leo, C. van Breemen, C.H. Lee, Mechanism of ACh-induced asynchronous calcium waves and tonic contraction in porcine tracheal muscle bundle, Am. J. Phys. Lung Cell. Mol. Phys. 290 (2006) L459–L469.
[28] X.X. Chen, J.H. Zhang, B.H. Pan, H.L. Ren, X.L. Feng, J.L. Wang, et al., TRPC3-mediated Ca(2+) entry contributes to mouse airway smooth muscle cell proliferation induced by lipopolysaccharide, Cell Calcium 60 (2016) 273–281.
[29] T. Zhang, X.J. Luo, W.B. Sai, Yu MF, W.E. Li, Y.F. Ma, et al., Non-selective cation channels mediate chloroquine-induced relaxation in precontracted mouse airway smooth muscle, PLoS One 9 (2014) e101578.
[30] S.A. Doggrell, Bitter taste receptors as a target for bronchodilation, Expert Opin. Ther. Targets 15 (2011) 899–902.