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NS8593 inhibits Ca^{2+} permeant channels reversing mouse airway smooth muscle contraction

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ABSTRACT

Aims: This study focused on investigating whether NS8593 reverses airway smooth muscle (ASM) contraction and the underlying mechanism.
Main methods: ASM contraction in mouse tracheal rings and lung slices was measured. Currents mediated by voltage dependent Ca^{2+} channels (VDCCs) and ACH-activated channels were measured using the whole-cell patch-clamp technique in single tracheal smooth muscle cells (TSMCs). Intracellular Ca^{2+} level and cell length were measured using an LSM 700 laser confocal microscope and a Zen 2010 software. Mouse respiratory system resistance (Rrs) was assessed using a FlexiVent FX system.
Key findings: High K^+ (80 mM K^+) and ACH induced ASM contraction in mouse tracheal rings and lung slices, which was partially relaxed by nifedipine (blocker of L-type VDCCs, LVDCCs), YM-58483 (blocker of store-operated Ca^{2+} entry (SOCE), transient receptor potential C3 (TRPC3) and TRPC5 channels), respectively. However, the contraction was completely reversed by NS8593, whereas, slightly relaxed by formoterol. ACH activated inward currents, which displayed linear and reversed around 0 mV, indicating the currents were mediated by non-selective cation channels (NSCCs). Moreover, these currents were blocked by YM-58483. In addition, such currents were abolished by NS8593, implicating that NS8593 inhibits the same channels. Besides, NS8593 inhibited increases of intracellular Ca^{2+} and the associated cell shortening. Finally, NS8593 inhibited ACH-induced increases of mouse respirator system resistance (Rrs).
Significance: Our results indicate that NS8593 inhibits LVDCCs and NSCCs, resulting in decreases of intracellular Ca^{2+} and then leading to ASM relaxation. These data suggest that NS8593 might be a new bronchodilator.

1. Introduction

Allergic asthma is a chronic respiratory disease [6]. One of fundamental features is the exaggerated contraction of airway smooth muscle (ASM), which leads to excessive obstruction of the airway tract, limiting airflow [11,30,36]. Such enhanced ASM contraction can be induced by increases of intracellular Ca^{2+} [19]. One of sources is Ca^{2+} influx mediated by Ca^{2+} permeant channels, such as L-type voltage-dependent Ca^{2+} channels (LVDCCs) and store-operated Ca^{2+} entry (SOCE) channels [5,29], and transient receptor potential melastatin 7 (TRPM7) channels [31]. Therefore, these channels could be as targets for preventing the excessive contraction of ASM. Hence, we want to search an inhibitor of cation channels as a new potential bronchodilator. Because, the known bronchodilators, such as β_2 adrenergic agonists, have side

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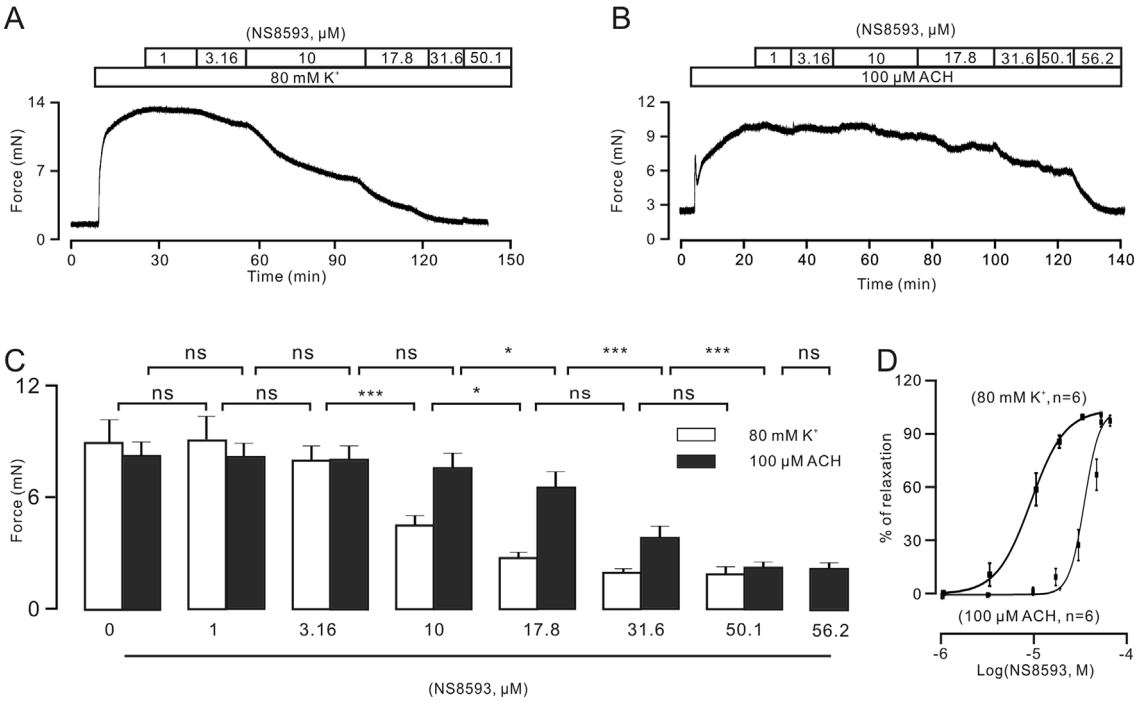

Fig. 1. NS8593 reverses contraction of mouse tracheal smooth muscle. **(A–B)** High K^+- and ACH-induced contraction in a tracheal ring (TR) was reversed by NS8593 (*left*). **(C–D)** The summary results of NS8593-induced relaxations. ns: not significant, * P < 0.05, *** P < 0.001.

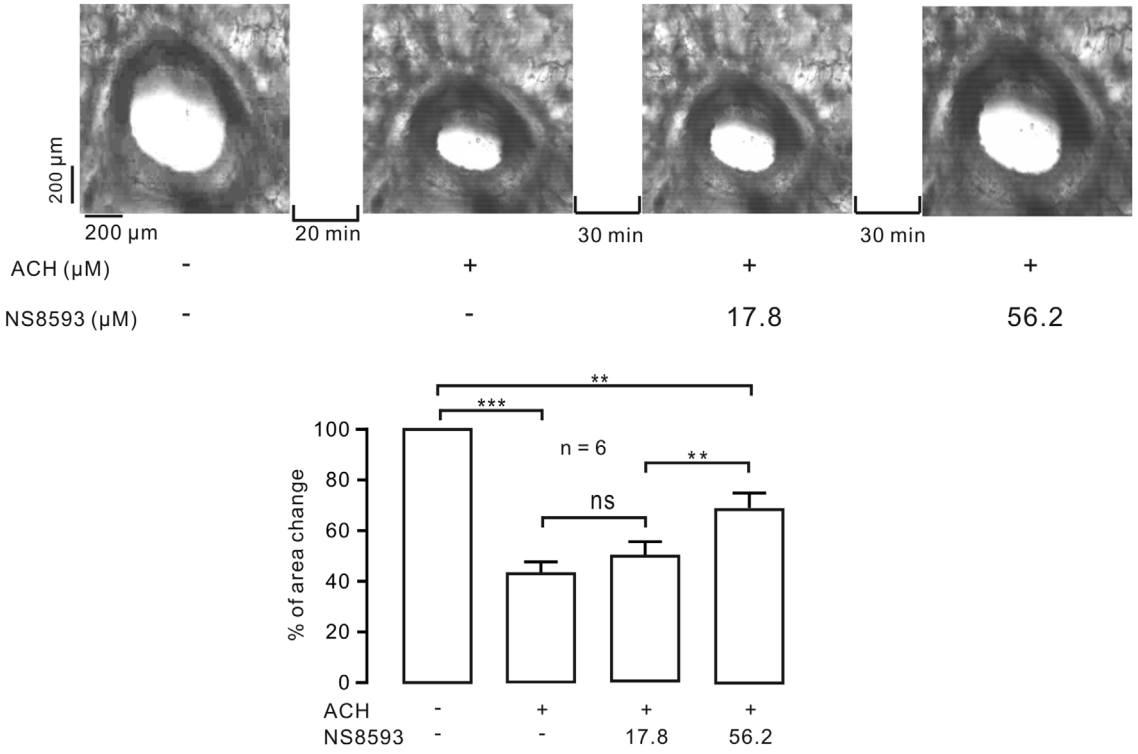

Fig. 2. NS8593 reverses ACH-induced contraction in bronchial smooth muscle. **(A)** The area of the airway lumens in a lung slice decreased following the application of ACH, however, which reversed by NS8593. **(B)** The summary results. ** P < 0.01, *** P < 0.001.

effects, including desensitization, palpitations, tremor, headache, cardiovascular death, ischemic heart disease, cardiac failure and airway inflammation enhancement [1,26].

TRPM7 channels are Ca^{2+} permeable channels [31]. Thus, when they are blocked by a selective blocker, NS8593 [8], intracellular Ca^{2+} will decrease [3,12]. These data suggest that NS8593 could result in ASM relaxation through reducing cytosolic Ca^{2+} level. On the other hand, NS8593 can inhibit small conductance Ca^{2+}-activated K^+ channels [34] through interacting with gating structures within the inner pore vestibule [17]. Therefore, NS8593 might enable to induce ASM contraction, on the basis of that the blockade of these channels would result in depolarization, which then activates LVDCCs, leading to

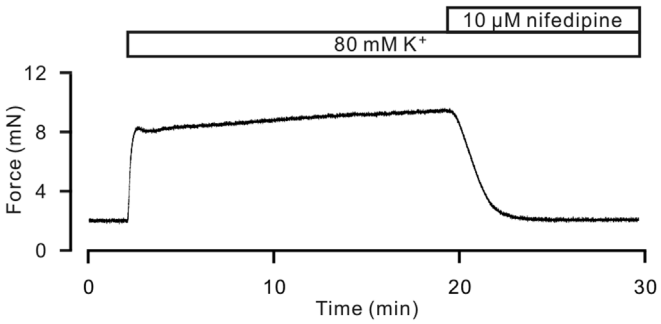

Fig. 3. Effect of nifedipine on high K^+-induced ASM contraction in tracheal rings. A representative of six independent experiments shows that high K^+-induced contraction was completely reversed by LVDCC selective blocker nifedipine, suggesting that the high K^+-induced contraction is totally due to the opening of LVDCCs.

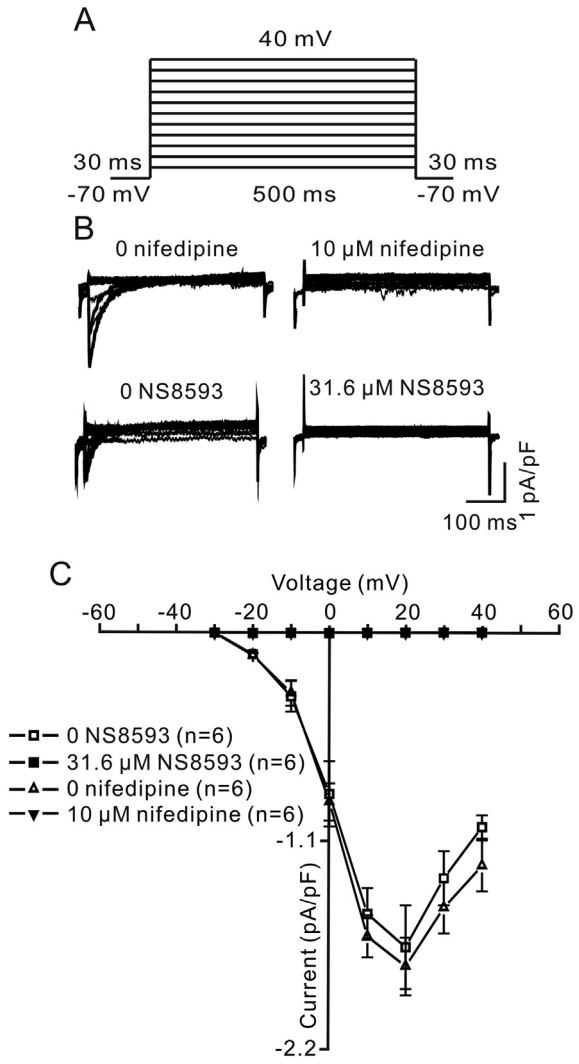

Fig. 4. NS8593 blocks LVDCC-mediated currents in TSMCs. (**A**) The protocol used to measure LVDCC currents. (**B**) The currents were completely abolished by nifedipine and NS8593. (**C**) The current-voltage curves. These results demonstrate that NS8593 inhibits LVDCCs.

Ca^{2+} entry and contraction.

Collectively, the effect of NS8593 on ASM tension is uncertain. In this study, we found that NS8593 reversed ASM contraction *in vitro and in vivo* via inhibiting cation channels.

2. Materials and methods

2.1. Animals

Six-eight-week male BALB/c mice were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China) and were raised in a standard animal facility. Animal experiments were conducted in accordance with the guidelines and the protocols approved by the Institutional Animal Care and Use Committee of the South-Central University for Nationalities.

2.2. Reagents

Fluo-4AM was purchased from Invitrogen (Eugene, OR, USA); NS8593, acetylcholine chloride (ACH), papain, collagenase H, dithiothreitol (DTT), bovine serum albumin (BSA), nifedipine, niflumic acid, YM-58483, Mg-ATP, tetraethylammonium chloride (TEA) and ovalbumin (OVA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Formoterol was purchased from Targetmol (Shanghai, China); The others were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Fluo-4AM, nifedipine, niflumic acid, YM-58483, NS8593 and formoterol were dissolved in DMSO.

2.3. Mouse model of allergic asthma

The model was prepared as previously described [35]. Mice were sensitized by intraperitoneal injection of 0.2 mL of 3 mg/mL OVA with 15 mg/mL $Al(OH)_3$ on day 1 and day 8. From day 15 through day 19, the animals were daily challenged once by intranasal instillation of 50 μL of 3 mg/mL OVA. Control animals were identically treated with the vehicles.

2.4. Measurement of respiratory system resistance

Mouse respiratory system resistance (Rrs) was measured using a FlexiVent FX system (SCIREQ Inc., Canada) as previously described [10]. Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (70 mg/kg). The tracheae were exposed and intubated with 18-G metal cannulas. The cannulas were then connected to the FlexiVent system.

To assess the effect of ACH on Rrs, ACH was inhaled for 30 s and the real time values of Rrs were then acquired for 120 s. The values were averaged and which was use to represent Rrs. Concentrations of ACH included 3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL, 50 mg/mL. The time interval between two concerntrations was 4 min. Based on the averaged Rrs values and ACH concentrations, we constructed Rrs-ACH curves. When observing the effect of NS8593, it was inhaled with ACH.

2.5. Measurement of ASM contraction

Tension of ASM was measured in mouse tracheal rings using BL-420 system (Taimeng, Chengdu, China) [37,39]. Mice were killed by intraperitoneally injecting 150 mg/kg sodium pentobarbital. The tracheae were obtained and placed in ice-cold physiological salt solution (PSS) including (mM): 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 D-glucose (pH = 7.40). Tracheal rings (approximately 5 mm) were cut above the bifurcations, which were then suspended in organ chambers filled with 37 °C PSS bubbling with 95% O_2, 5% CO_2. A preload of 300 mg was added on each sample. The samples were equilibrated for 60 min, and were then contracted for three times with high K^+ (80 mM K^+) or ACH (100 μM). After then, they rested for 30 min and experiments were performed.

Bronchial smooth muscle contraction was measured in mouse lung slices [2,18]. Mice were killed by 150 mg/kg sodium pentobarbital. The tracheae were exposed and cannulated. Agarose gel (2%, ~1.3 mL,

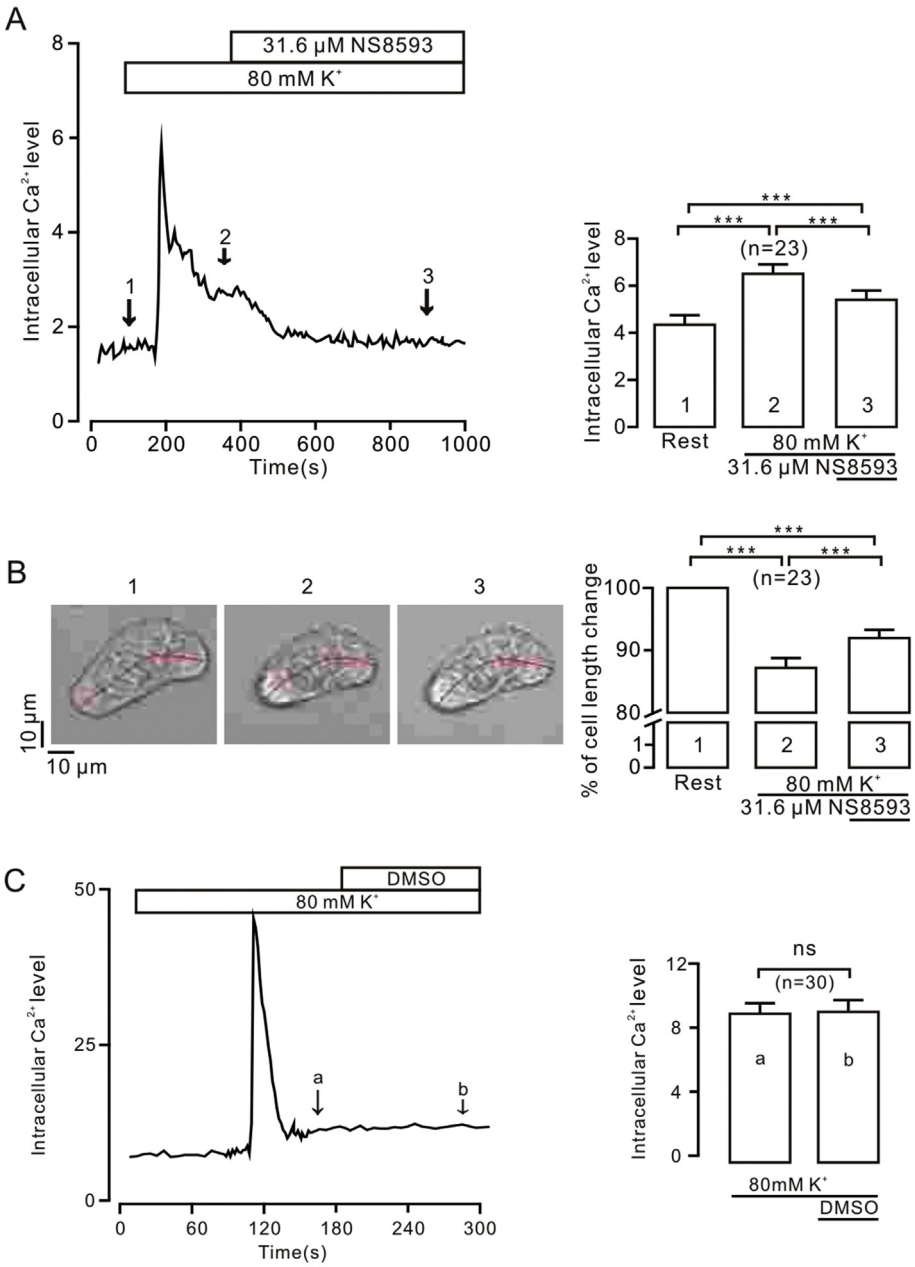

Fig. 5. NS8593 inhibits high K^+-induced Ca^{2+} elevation and cell shortening in TSMCs. (**A, B**) High K^+ induced sustained increases of intracellular Ca^{2+}, which associated with cell shortening. They both were inhibited by NS8593. **(C)** The sustained increases of Ca^{2+} unchanged in the absence of NS8593. ns: not significant, *** $P < 0.001$. These data indicate that NS8593 relaxes ASM via inhibiting LVDCC-mediated Ca^{2+} influx.

37 °C) was slowly injected into the lungs. After gel gelatinized, the lungs were taken and kept at 4 °C Hanks' balanced salt solution (HBSS) for 30 min. HBSS included (mM): 137.93 NaCl, 5.33 KCl, 4.17 $NaHCO_3$, 1.26 $CaCl_2$, 0.493 $MgCl_2$, 0.407 $MgSO_4$, 0.4414 KH_2PO_4, 0.338 $Na_2HPO_4{\cdot}12H_2O$, and 5.56 D-glucose, 20 HEPES (pH = 7.40). Slices of 350 μm thick were cut using a VT1200 (Leica Microsystems GmbH, Wetzlar, Germany) and placed in wells of 24-well plates containing 2 mL DMEM medium (GIBICO). The plates were then maintained in an incubator (37 °C, 95% O_2, 5% CO_2) for 3 h. Slices were then fixed in a chamber with a small nylon mesh and perfused with HBSS at a rate of ~800 μL/min. Images of the slices under 10× objective lens were acquired at a rate of 30 frames/min using an LSM 700 laser confocal microscope (Carl Zeiss, Göttingen, Germany). The cross-sectional areas of the airway lumens were measured using a Zen 2010 software (Carl Zeiss, Göttingen, Germany). A decrease and an increase of the airway lumen areas represent ASM contraction and relaxation, respectively. Experiments were performed at room temperature.

2.6. Isolation of single tracheal smooth muscle cells

Single mouse tracheal smooth muscle cells (TSMCs) were isolated as the described method [39]. Briefly, tracheal muscle was digested in low Ca^{2+} solution (LCS) contained 2 mg/mL papain for 25 min at 35 °C, and then in LCS supplemented 1 mg/mL collagenase H for 7 min at 35 °C. The muscle was then washed three times with LCS contained 1 mg/mL BSA, and then triturated to release single TSMCs. The cells were stored at 0 °C and used in the experiments of channel current recordings and cytosolic Ca^{2+} measurements. LCS contained (mM): 136 NaCl, 5.36 KCl, 20 HEPES, 11 D-glucose, 0.44 KH_2PO_4, 0.34 $Na_2HPO_4{\cdot}12H_2O$, 4.16 $NaHCO_3$ (pH = 7.10).

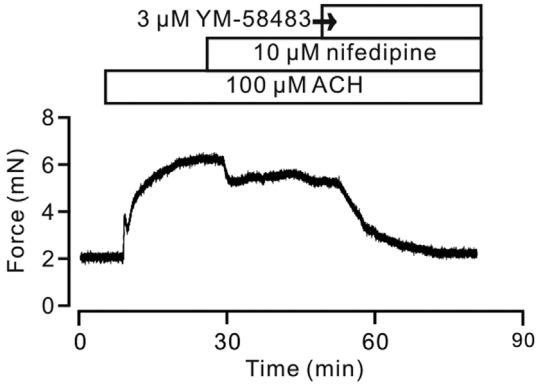

Fig. 6. Effects of nifedipine and YM-58483 on ACH-induced contraction in mouse tracheal rings. A representative of six independent experiments shows that ACH-induced sustained contraction was reversed by nifedipine and YM-58483, respectively, indicating that LVDCCs and SOCE play a role in ACH-induced contraction.

2.7. Recording of channel currents

The conventional whole-cell patch-clamp technique was employed to record LVDCC-mediated currents and ACH-activated currents in single TSMCs with an EPC-10 amplifier and a PatchMaster software (HEKA, Lambrecht, Germany) [25].

LVDCC currents were measured using Ba^{2+} as charge carriers. The pipette solution contained (mM): 130 CsCl, 10 EGTA, 4 $MgCl_2$, 4 Mg-ATP, 10 HEPES (pH = 7.40). The extracellular solution included (mM): 107 NaCl, 1 $MgCl_2$, 27.5 $BaCl_2$, 11 D-glucose, 10 HEPES, 10 TEA-Cl (pH = 7.40). Cells were patched and held at −70 mV. Currents were triggered by a 500 ms depolarization step from −70 mV to 40 mV with a 10 mV-increment every 10 s.

ACH-activated currents were measured with a 500 ms ramp from −80 mV to 60 mV. The values at −70 mV represent ACH-induced currents. The pipette solution was (mM): 18 CsCl, 108 cesium acetate, 1.2 $MgCl_2$, 10 HEPES, 3 EGTA, 1 $CaCl_2$ (pH = 7.20; ~70 nM free Ca^{2+}, WEBMAXC STANDARD, http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm). The bath solution was K^+-free PSS containing 10 μM nifedipine, 100 μM niflumic acid, and 10 mM TEA (to isolate NSCC currents by blocking Ca^{2+}, Cl^- and K^+ currents, respectively).

2.8. Measurement of intracellular Ca^{2+} level and cell length

Intracellular Ca^{2+} levels were measured using the LSM 700 laser confocal microscope and the Zen 2010 software [24,39]. Cells were loaded with Fluo-4AM for 20 min in a chamber at room temperature, and then were washed for 5 min with PSS bubbling with 95% O_2/5% CO_2. The wavelength of the excited lights was 488 nm. The emission lights were filtered with a 505 nm filter and then to generate images. The values of the fluorescent intensity reflect the levels of intracellular Ca^{2+}.

The lengths of the cells were simultaneously recorded using transmitted lights provided by the LSM 700 laser confocal microscope, which were then measured using the Zen 2010 software.

2.9. Statistical analysis

Data are expressed as mean ± SEM. Comparisons between two groups were performed with the Students t-test and among multiple groups by the one-way ANONA. Difference was considered significant at $P < 0.05$.

3. Results

3.1. NS8593 reverses contraction of mouse ASM

We first investigated the effect of NS8593 on high K^+- and ACH-induced contraction in tracheal rings (TRs). As shown in Fig. 1A, B, NS8593 reversed the contractions in a dose-dependent manner. The results from six identical experiments were summarized and presented in Fig. 1C, D, respectively. The IC_{50} value and maximal inhibition of NS8593 on high K^+- and ACH-caused contraction were 8.9 ± 1.3 μM and 99.9 ± 1.7% (n = 6), 39.8 ± 1.0 μM and 98.5 ± 2.9% (n = 6), respectively.

The control vehicle (DMSO) did not affect high K^+- and ACH-induced contraction (n = 6 for each group, data not shown). Besides, NS8593 failed to change the tone in resting TRs (n = 4, data not shown).

We next investigated whether NS8593 has similar reversal action on bronchial smooth muscle contraction. As shown in Fig. 2, the areas of the airway lumens in lung slices decreased following the addition of 100 μM ACH, however, which were partially reversed by 39.8 μM (the IC50 value) and 56.2 μM (maximal inhibitory concentration) NS8593.

Taken together, NS8593 reverses ASM contraction.

3.2. Mechanism of NS8593-induced relaxation

High K^+ induces cell membrane depolarization that activates voltage-dependent Ca^{2+} channels (VDCCs), allowing extracellular Ca^{2+} influx, subsequently leading to contraction of ASM [38,39]. Our results showed that high K^+-induced contraction was reduced by 99.3 ± 0.9% (n = 6) following the addition of nifedipine, a selective blocker of L-type VDCCs (LVDCCs) (Fig. 3). These results indicate that high K^+-induced contraction is completely mediated by LVDCCs. Therefore, the relaxation of ASM caused by NS8593 might be due to inhibition of LVDCCs.

We next studied whether NS8593 inhibits LVDCCs. VDCC-mediated currents were measured in single TSMCs, which were completely blocked by nifedipine (Fig. 4). These results indicate that the currents are mediated by LVDCCs. The same currents were abolished by NS8593, suggesting that NS8593 inhibits LVDCCs.

We then investigated whether LVDCCs result in intracellular Ca^{2+} increases and whether which are affected by NS8593 in single TSMCs. Following activation of LVDCCs by high K^+, transient and sustained intracellular Ca^{2+} increases and cell shortening were observed. However, the sustained Ca^{2+} elevations and the cell shortening were inhibited by 31.6 μM NS8593 (Fig. 5A, B). Without adding NS8593, the sustained increases of Ca^{2+} did not decrease (Fig. 5C). Moreover, high K^+ failed to induce Ca^{2+} increases in NS8593-incubated 55 cells (data not shown). These results indicate that NS8593 inhibits LVDCCs, resulting in the termination of Ca^{2+} influx and then leading to relaxation.

For ACH-induced ASM contraction, LVDCCs and SOCE play an important role [32,38]. Thus, we observed the effects of their inhibitors on ACH-induced TR contraction. As shown in Fig. 6, nifedipine and YM-58483 partially reversed the contraction. The relaxation was 13.2 ± 1.4% (n = 6) and 77.4 ± 1.9% (n = 6), respectively. YM-58483 is not only an inhibitor of SOCE but also a blocker of TRPC3 and TRPC5 channels [15,27,28]. Therefore, these results suggest that LVDCCs and SOCE play a role in ACH-induced contraction, and that TRPC3 and/or TRPC5 would function as SOCE. Thus, NSCC channels would involve in NS8593-induced relaxation.

We next observed whether NS8593 inhibits ACH-activated channels. As shown in Fig. 7, in the presence of three inhibitors TEA, nifedipine and niflumic acid, ACH activated currents and which were totally blocked by YM-58483 (from −7.8 ± 0.8 pA to −0.02 ± 0.20 pA, n = 6). The same currents were also completely blocked by NS8593 (from −7.9 ± 0.6 pA to −0.5 ± 0.1 pA, n = 6). Moreover, the currents were linear and their reversal potentials were

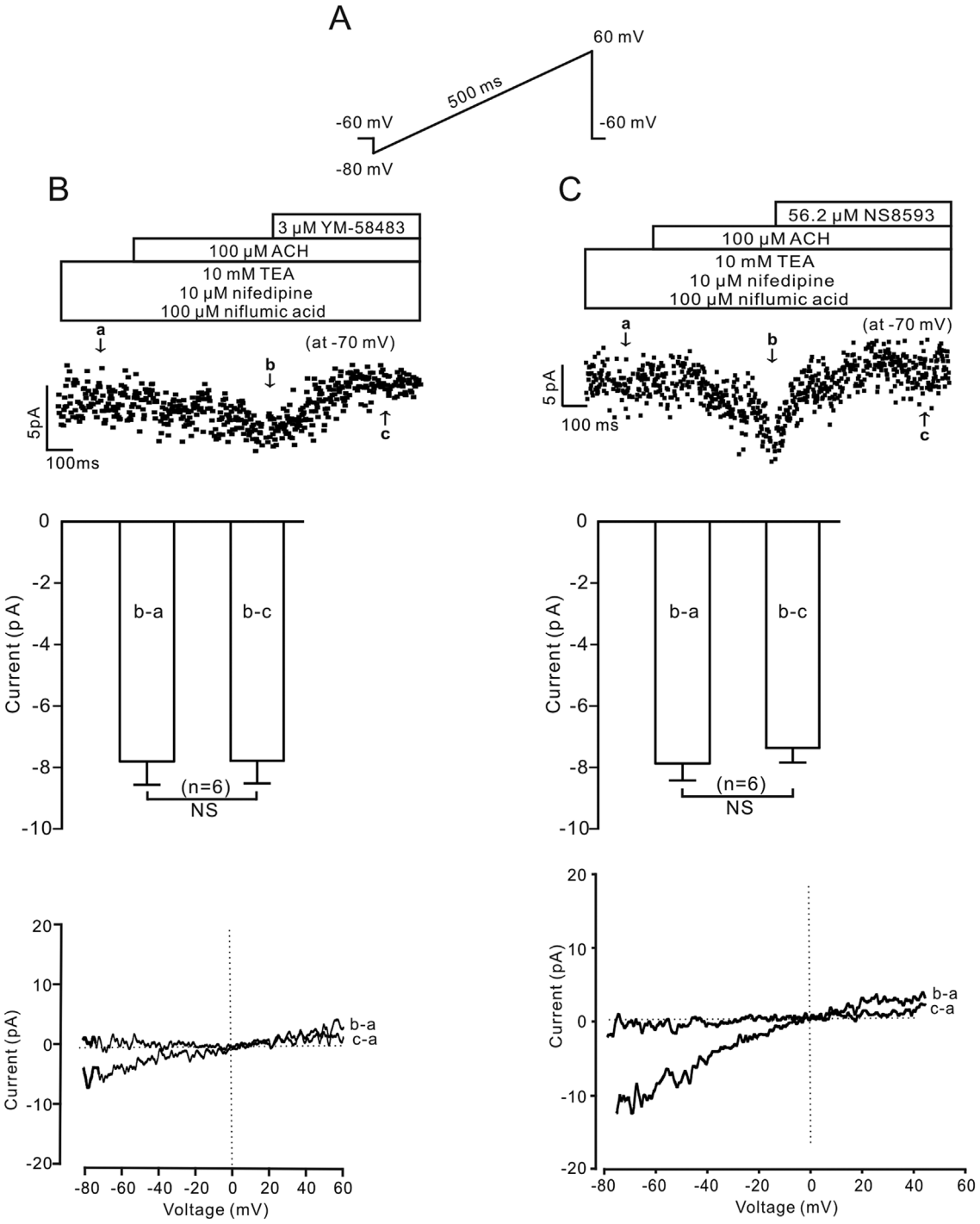

Fig. 7. NS8593 blocks ACH-activated currents in TSMCs. **(A)** The ramp used to measure ACH-activated currents. **(B)** In the presence of TEA, niflumic acid and nifedipine, ACH-induced currents at −70 mV were inhibited by YM-58483. **(C)** The same currents were inhibited by NS8593. In addition, the currents displayed linear and reversed around 0 mV. ns: not significant. These data indicate that ACH-activated channels are NSCCs, which are inhibited by NS8593.

close 0 mV, suggesting these currents are mediated by NSCCs. These results indicate that ACH-activated channels are NSCCs, which might be TRPC3 and/or TRPC5 based on that the currents were sensitive to YM-58483, these channels are inhibited by NS8593.

Furthermore, NS8593 inhibited ACH-induced sustained intracellular Ca^{2+} increases and the associated cell shortening (Fig. 8A, B). Whereas, the sustained Ca^{2+} increases did not change in the absence of NS8593 (Fig. 8C). These data suggest that NS8593 blocks Ca^{2+} influx mediated by SOCE (as well as LVDCCs).

We next studied whether *in vivo* NS8593 inhibits ASM contraction. As shown in Fig. 9, ACH induced increases of Rrs in mice, however, in the presence of NS8593, the increases were inhibited.

We further investigated whether NS8593 reverses contraction of ASM from models of allergic asthma. As shown in Fig. 10, high K^+- and ACH-elicited contraction in TRs from model mice were reversed by NS8593. The IC50 and maximal relaxation were 16.4 ± 1.5 μM and 95.0 ± 2.8% (n = 6), 32.2 ± 2.9 μM and 95.3 ± 1.6% (n = 6), respectively. These results indicate that NS8593 has similar inhibitory action in asthmatic ASM.

In order to revalue the efficacy of NS8593, we studied the relaxant

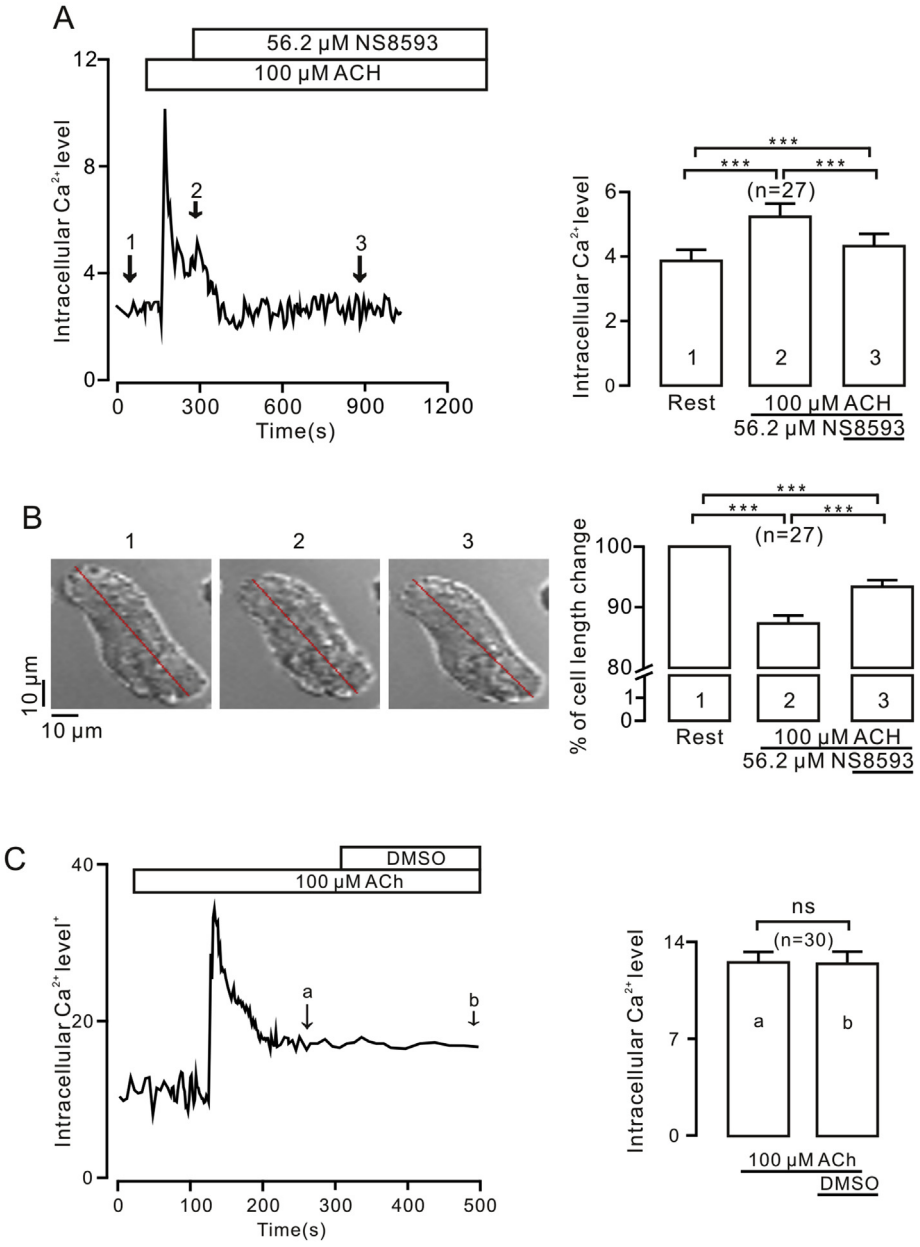

Fig. 8. NS8593 inhibits ACH-induced Ca^{2+} elevation and cell shortening in TSMCs. **(A, B)** ACH-elicited sustained increases of intracellular Ca^{2+} and associated cell shortening were inhibited by NS8593. **(C)** The increases of intracellular Ca^{2+} did not change in the absence of NS8593. ns: not significant, *** $P < 0.001$. These results suggest that NS8593-induced relaxation is owing to inhibition of ACH-activated channels.

action of formoterol, a known bronchodilator. Formoterol slightly reversed ACH-induced contraction in TRs (Fig. 11). The IC50 and maximal relaxation were 0.102 ± 0.036 μM and 23.4 ± 1.6% (n = 6), respectively.

4. Discussion

In this study, our results show that *in vitro and in vivo* NS8593 reversed high K^+- and ACH-induced contraction of tracheal and bronchial smooth muscle from healthy and allergic asthma model mice. The reversal action of NS8593 was stronger compared to the known bronchodilator formoterol. High K^+- and ACH-caused contraction was also relaxed by nifedipine, a blocker of LVDCC, and YM-58483, an inhibitor of SOCE, TRPC3 and TRPC5. In addition, high K^+ and ACH elicited intracellular Ca^{2+} increases and the associated cell shortening, however, both were inhibited by NS8593. Moreover, NS8593 blocked the currents mediated by LVDCCs and ACH-activated NSCCs that sensitive to YM-58483. Finally, NS8593 inhibited ACH-caused airway resistance increases *in vivo*. These data suggest that NS8593 reverses ASM contraction through inhibiting LVDCCs and NSCCs (such as TRPC3 and TRPC5).

ASM cells express TRPM7 channels [23], which permeate Ca^{2+} [31]. Thus, the activated TRPM7 could result in muscle contraction. In contrast, to block TRPM7 channels would induce relaxation. These were demonstrated by that NS8593, a blocker of TRPM7 channels, reversed high K^+- and ACH-induced ASM contraction, regardless of ASM from controls and allergic asthma mice, airway segments, *in vitro and in vivo* (Figs. 1–2, 5, 8-10). Collectively, these results indicate that NS8593 might be a new bronchodilator for the treatment of asthma. However, its inhibitory action likely was not due to inhibition of TRPM7 channels, because high K^+-induced contraction was completely blocked by nifedipine, a selective blocker LVDCCs (Fig. 3), suggesting that LVDCCs, not

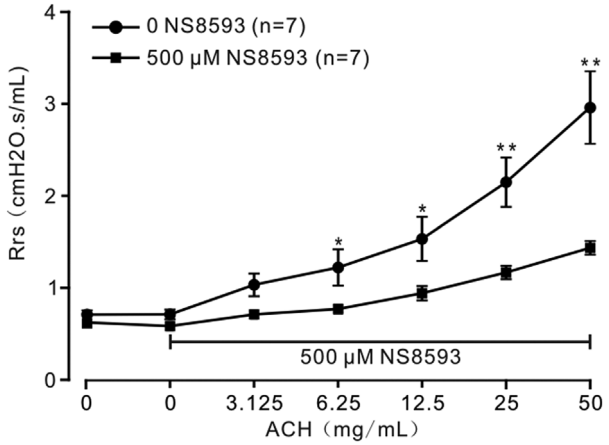

Fig. 9. NS8593 inhibits Rrs. ACH-induced increases of Rrs in mice were inhibited by NS8593. * P < 0.05, ** P < 0.01.

TRPM7, mediate the contraction. Thus, NS8593 reversed high K^+-induced contraction might be owing to its inhibition on LVDCCs (Fig. 1A). Indeed, this was confirmed by that NS8593 inhibited LVDCC-mediated currents (Fig. 4). Therefore, NS8593-induced relaxation might result from inhibiting LVDCC-mediated Ca^{2+} entry. This was testified by that NS8593 inhibited intracellular Ca^{2+} decreases and cell shortening in single TSMCs (Fig. 5). Taken together, our results indicate that NS8593 inhibits LVDCCs, leading to the end of Ca^{2+} influx and then inducing relaxation.

However, nifedipine can block T-type VDCCs [20], unknown non-selective store-operated cation channels [9], and K^+ channels, such as Kv1.5 [22,40], Kv2.1 [21], Kv4.2 [4], transient outward channel [16] and ultra-rapid delayed rectifier channel [13]. The T-type VDCCs and the unknown cation channels will induce contraction because they can result in Ca^{2+} influx and depolarization (that then activates LVDCCs, causing contraction). In contrast, the activation of the K^+ channels can induce in hyperpolarization, which will then lead to inactivation of LVDCCs, resulting in decreases of intracellular Ca^{2+} to cause relaxation. Thus, if these channels played a role in ACH-induced ASM contraction, they would involve in nifedipine- and NS8593-induced relaxation. However, we do not think these would be the dominated mechanism.

In addition, NS8593 reversed ACH-induced contraction (Figs. 1, 2). While, such contraction was partially reversed by LVDCC blocker nifedipine, and SOCE, TRPC3 and TRPC5 inhibitor YM-58483 (Fig. 6). These results suggest that LVDCCs are also involved in ACH-induced contraction, and that these channel- and SOCE-mediated Ca^{2+} influx exerts a crucial role. Thus, NS8593 might be through inhibiting these pathways to reduce cytosolic Ca^{2+} and then result in relaxation. In fact, NS8593 inhibited LVDCCs and then induced relaxation had been observed as above described. To further define the underlying pathway of SOCE, we recorded currents and investigated which types of channels mediated the currents. We isolated ACH-induced currents, which displayed linear and reversed around 0 mV (Fig. 7). These features suggest that the currents were mediated by NSCCs. Thus, YM-58483-induced relaxation could be partially attributed to its inhibition on these NSCCs. On the basis of that YM-58483 is an inhibitor of SOCE, TRPC3 and TRPC5, YM-58483-inhibited NSCCs will be TRPC3 and/or TRPC5. These results exclude the involvement of STIM/Orai channels, which function as SOCE [33]. The reason is that these channel-mediated currents inwardly rectify and reverse at more positive potential [14]. In addition, the involvement of TRPC3 and/or TRPC5 would be supported by that these channels are activated by Ca^{2+} store depletion, and which was observed (Figs. 5, 8).

TRPC3 and TRPC5 channels mediate divalent and monovalent cation (such as Ca^{2+}, Na^+) entry. Entered Na^+ can induce depolarization resulting in intracellular Ca^{2+} increases through activating other channels, such as LVDCCs. Thus, we observed sustained increases of Ca^{2+} (Figs. 5, 8), underlying ACH-induced sustained contraction. NS8593 blocked the currents sensitive to YM-58483 (Fig. 7), suggesting NS8593 inhibits TRPC3 and/or TRPC5. Therefore, following the inhibition of these channels by NS8593, intracellular Ca^{2+} declined and ASM cells then relaxed (Fig. 8).

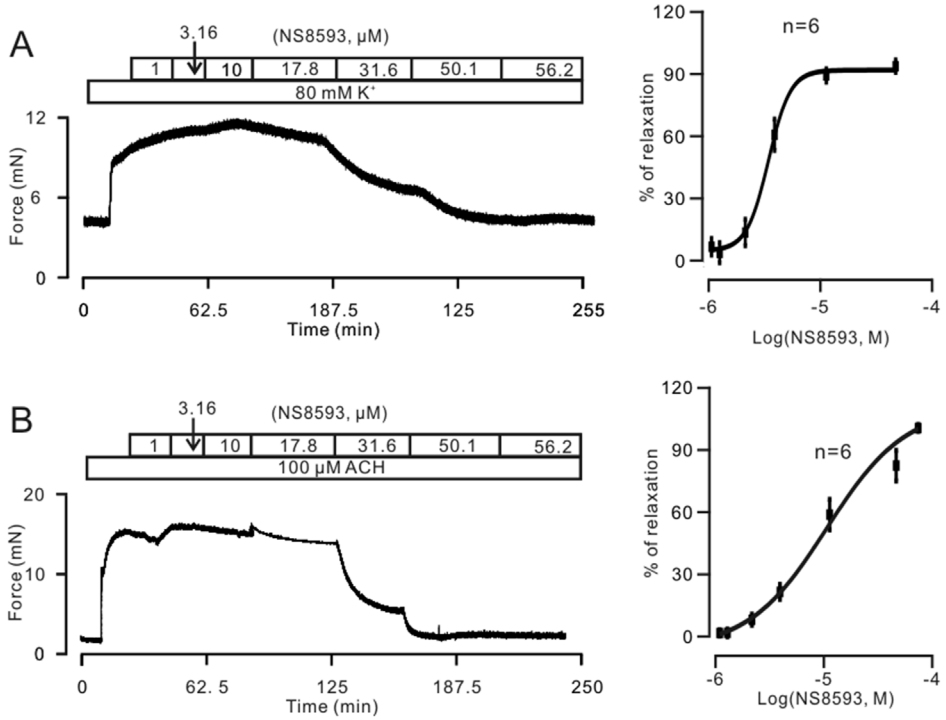

Fig. 10. NS8593 reverses contraction of asthmatic ASM. **(A, B)** High K^+- and ACH-induced contraction in TRs from mouse models of allergic asthma was reversed by NS8593.

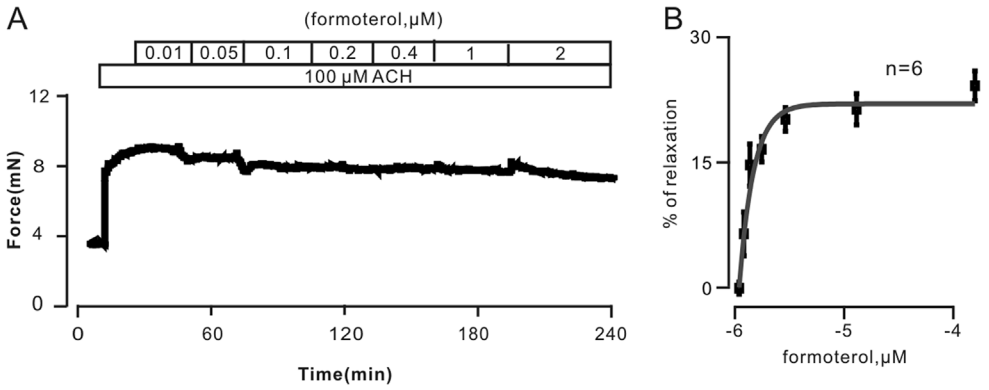

Fig. 11. Effects of formoterol on ASM contraction. **(A–B)** ACH-induced contraction in TRs was slightly reversed by formoterol.

Besides, nifedipine induced relaxation was smaller than that caused by YM-58483 (Fig. 6). This result implies that LVDCCs play a less role compared to SOCE, consistent with that low concentration MCh-induced intracellular Ca^{2+} oscillations are not blocked by LVDCC blocker nifedipine although the frequency of the oscillations is reduced, however, the oscillations are abolished by SOCE blocker [5,7].

Finally, NS8593 inhibited ACH-induced Rrs increases (Fig. 9). These results suggest that NS8593 has a relaxant action *in vivo*, consistent with the *in vitro* results (Figs. 1, 2). However, Rrs reflects contraction of ASM from entire airways, thus, it is possible that tracheal and bronchial smooth muscle might not have similar relaxation in response to NS8593. Indeed, the *in vitro* experiments showed that NS8593 failed to completely reverse the contraction in lung slices compared to TRs (Figs. 1, 2). These results likely indicate that NS8593 has less effect in large airway smooth muscle compared to small airway smooth muscle. However, we rather believe it would be due to that the adhesive force between the slice and coverslip glass prevented the slice to completely return to the original position after ASM complete relaxation. In addition, NS8593 can reverse the contraction of ASM from allergic asthma models (Fig. 10). All these results suggest that NS8593 might be a bronchodilator for the treatment of asthma. Moreover, it might be a potent bronchodilator based on that it can totally reverse the contraction, however, the known bronchodilator formoterol slightly relaxed the contraction (Fig. 11). However, the relaxant effect of NS8593 needs to be further testified in human airway smooth muscle.

5. Conclusion

In summary, our results indicate that NS8593 potently relaxes healthy and asthmatic ASM contraction via inhibiting cation channels. These results suggest that NS8593 might be a new bronchodilator.

Declaration of competing Interest

The authors declare no conflict of interest.

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