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Benidipine, an anti-hypertensive drug, relaxes mouse airway smooth muscle

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ABSTRACT

Aims: Benidipine is a dihydropyridine (DHP) derived Ca^{2+} antagonist, can block triple Ca^{2+} channels (L, N, and T). It has been used as an safety anti-hypertensive drug because of its long-acting relaxant effect on vascular smooth muscle (VSM). However, whether benidipine has similar pharmacological actions in airway smooth muscle (ASM) is unknown. This research aims to reveal the relaxant property and Ca^{2+} antagonistic effect of benidipine on ASM.

***Main methods*:** The relaxant property of mouse ASM was investigated by tissue tension tests, and Ca^{2+} antagonistic effect was evaluated through patch-clamp techniques.

Key findings: Benidipine caused dose-dependent relaxations on high K^{+} (80 mM) induced precontraction in mouse ASM, which relied on inhibition of extracellular Ca^{2+} influx, and 1 μM benidipine totally blocked L-type voltage-dependent Ca^{2+} channels (LVDCCs) currents in airway smooth muscle cells (ASMCs). Benidipine also showed dose-dependent inhibition of ACh-induced precontraction with or without the LVDCCs blocker nifedipine, and 100 μM benidipine blocked ACh-stimulated Ca^{2+} influx through not only LVDCCs but also non-selective cation channels (NSCCs).

Significance: Benidipine blocked LVDCCs and NSCCs to abolish these channels-mediated Ca^{2+} influx, which relaxed precontracted ASM. This study represented benidipine with a new potential medicinal value for ASM hypercontractility.

Keywords: benidipine; airway smooth muscle; relaxation; L-type voltage-dependent

Ca^{2+} channels; non-selective cation channels; Ca^{2+} influx.

1. Introduction

Asthma is a common chronic inflammatory disease of airways, affects over 300 million people globally and causes about 300 thousand deaths per year [1-3]. It is characterized by airway obstruction, airway hyperresponsiveness (AHR) and airway remodeling [4]. Dysfunction of airway smooth muscle (ASM) plays a critical role in asthma pathogenesis and especially contributes to contractility alteration and airway narrowing [5]. At present, inhaled β2-agonists combined with glucocorticoids are commonly used for the relief of asthmatic symptoms. However, the adverse effects of β2-agonists are obvious, such as palpitation, tremor, headache, cardiovascular death, and cardiac failure [6]. It is urgent to find effective and safety drugs for the treatment of ASM dysfunction. All contractile stimulations of ASM (such as depolarization or receptor-mediated) will finally result in increased intracellular Ca^{2+} concentration or/and Ca^{2+} sensitivity of contraction [7-9]. So looking for drugs which block Ca^{2+} channel or sensitization pathways may be a good solution to relieve ASM dysfunction.

Benidipine (or benidipine hydrochloride, KW-3049), is one of the second-generation dihydropyridine (DHP) Ca^{2+} antagonists and developed in Japan. It blocks L-, N-, and T-type triple Ca^{2+} channels through a membrane approach [10, 11]. Benidipine exerts potent, long-lasting antihypertensive activity because of its high affinity for the DHP binding site on vascular L-type Ca^{2+} channels, inhibition of tachycardia through T-type Ca^{2+} channels and suppressing the release of catecholamines from sympathetic nerve through N-type Ca^{2+} channels [12]. The toxicity studies and clinical reports suggest that benidipine is a highly safe drug at a wide range of dose levels and with long-acting pharmacological effects [13, 14].

Besides, benidipine has high vascular selectivity and suppresses VSM proliferation [15, 16]. However, until now the pharmacological actions of benidipine in ASM is unknown.

In this study, we investigated benidipine relaxation effects on mouse ASM by tissue tension tests and single cell ion channel currents recording. The results show that benidipine could both relax high K^+ and ACh induced precontraction in a dose-dependent manner through LVDCCs- and NSCCs-mediated Ca^{2+} influx.

2. Materials and methods

2.1 Reagents

Nifedipine, acetylcholine (ACh), bovine serum albumin (BSA), papain, collagenase H, dithiothreitol (DTT), cesium hydroxide (CsOH), cesium chloride (CsCl), niflumic acid (NA), pyrazole 3 (Pyr3), gadolinium, adenosine 5'-triphosphate magnesium salt (Mg-ATP), and tetraethylammonium chloride (TEA-Cl) were purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). Benidipine and histamine were purchased from Selleckchem (Houston, TX, USA). All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Nifedipine, histamine and benidipine were dissolved in dimethyl sulfoxide (DMSO) and others in corresponding solutions used in the experiments.

2.2 Animals

Six-week male BALB/c mice were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China) and were housed in SPF grade laboratory rooms under a 12 h light-12 h dark cycle. The animal studies were performed according to the guidelines of the Institutional Animal Care and Use

Committee of the South-Central University for Nationalities, and the corresponding protocol were approved by the Animal Care and Ethics Committee of the South-Central University for Nationalities (2017-SCUECAEC- 0268).

2.3 Measurement of ASM contraction

Mouse ASM contraction was measured in trachea rings (TRs) as previously described [17]. Briefly, the mice were sacrificed by cervical dislocation and the tracheas were isolated and quickly transferred to ice-cold PSS. TRs were cut and mounted in organ baths containing PSS bubbled with O_2 at 37°C. The resting tension was set to 300 mg. The experiments were initiated after the TRs were equilibrated for 60 min. Then, the TRs were stimulated with either 80 mM K^+ or 100 μM ACh, washed and then rested, repeated for 3 times. Following an additional 30 min rest, the ASM tension was measured under high K^+, ACh, benidipine, or particular channel inhibitors including nifedipine (10 μM), Pyr3 (30 μM), and gadolinium (30 μM). The high K^+ solutions contained KCl 80 mM.

2.4 Isolution of single ASMCs

Single mouse ASM cells were isolated as previously described [18]. Briefly, tracheas were isolated as described above in ASM dissociation buffer. The isolated tracheas were then incubated in ASM dissociation buffer containing papain, dithioerythritol and BSA at 35°C for 22 min. Then the tissues were transferred to ASM dissociation buffer containing collagenase H, dithioerythritol and BSA, incubated at 35°C for 8 min. At last the tissues were washed and gently triturated with 1mg/ml BSA to yield single ASMCs for use in subsequent patch-clamp experiments.

2.5 Recording of LVDCCs Currents

Whole-cell Ca^{2+} currents through LVDCCs were recorded using an EPC-10 patch-clamp amplifier (HEKA, Lambrecht, Germany). Ba^{2+} was used as a charge carrier. The pipette solution and external solution composition refer to previous report [19]. ASMCs were held at -70 mV. The currents were elicited following step depolarization for 500 ms from -70 to +40 mV with 10 mV increments every 1 s.

2.6 Recording of NSCCs currents

For the measurement of ACh-induced NSCCs currents, LVDCCs, Cl^- and K^+ currents were blocked by 10 μM nifedipine, 100 μM NA, and 10 mM TEA, respectively. The pipette solution and external solution composition refer to previous report [20]. ASMCs were patched in the classical whole-cell configuration with a holding potential of -60 mV. ACh-induced NSCCs currents were measured using a 500 ms ramp from -80 to +60 mV.

2.7 Statistical Analysis

Statistical analysis and significance were measured with Student's *t*-test using Origin 9.0 software. Data are expressed as the means ± SEM. Differences with $p < 0.05$ were defined significant.

3. Results

3.1 Benidipine relaxed high K^+-induced precontraction on mouse ASM

The high K^+-induced precontraction is widely used to assess the relaxant effects on different types of smooth muscle due to membrane depolarization and opening of LVDCCs [21-23]. In this study, we used high K^+ (80 mM)-induced precontraction as a standard contraction to test benidipine relaxant effect on mouse ASM. The results show that benidipine could totally relax high K^+-induced precontraction in a dose-dependent manner compared with vehicle (Fig. 1A and C); when 100 μM benidipine was added, the maximal relaxation reached to 99.61 ± 1.13%, and the half-maximal inhibitory (IC_{50}) concentration was 0.16 ± 0.02 μM by calculation (Fig. 1B). For convenience, we chose IC_{75} of approximately 1 μM to do the next high K^+ associated experiments. 10 μM nifedipine, a selective inhibitor of LVDCCs, as a positive control also performed a similar relaxation as benidipine (Fig. 1D). In addition, benidipine showed no effect on resting tension of ASM (Fig. 1E).

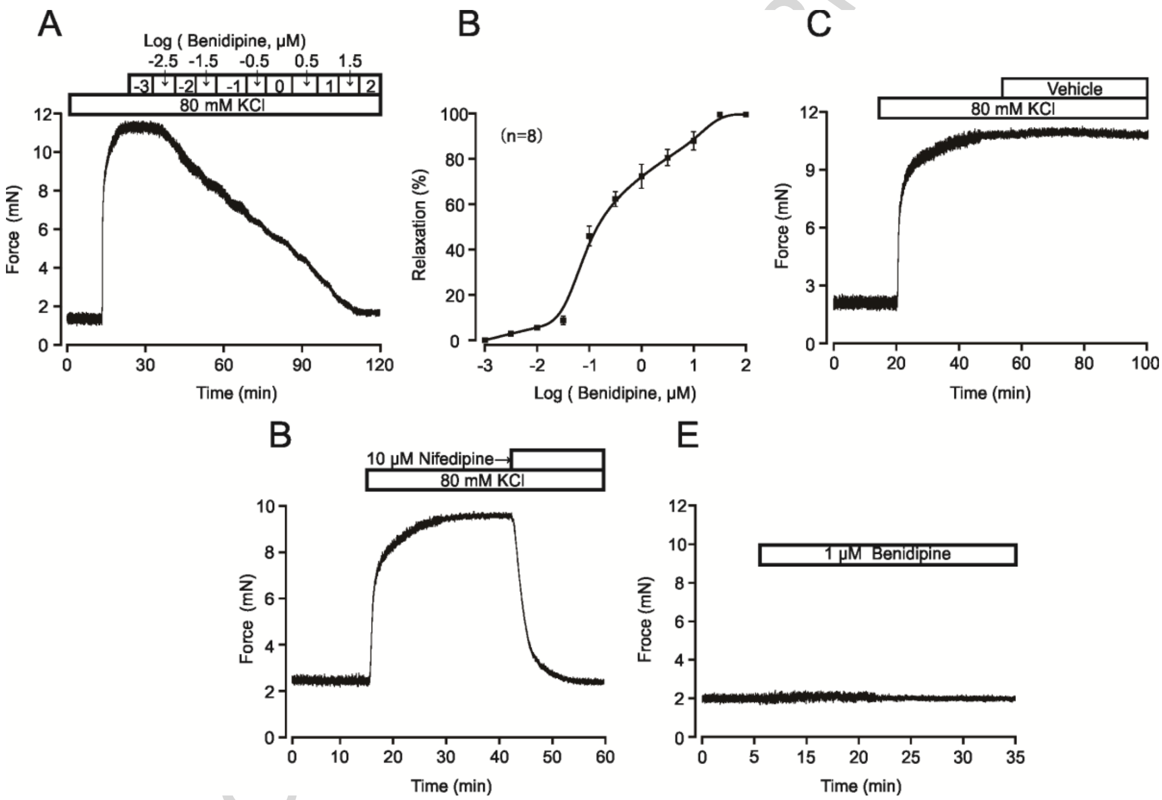

Fig. 1. Benidipine relaxed high K^+-induced precontraction on mouse ASM. (A) Benidipine caused dose-dependent relaxations on high K^+-induced precontraction; (B) Relaxation effect curve of benidipine (n = 8); (C) Vehicle (DMSO) had no relaxant effect on high K^+-induced precontraction; (D) Nifedipine performed a similar relaxtion as benidipine (n = 6); (E) Benidipine had no effect on resting tension (n = 6).

3.2 Benidipine blocked LVDCCs currents and high K^+-induced Ca^{2+} influx

High K^+-induced smooth muscle contraction is triggered by LVDCCs-mediated Ca^{2+} influx [21, 24]. To clarify whether benidipine relaxation effect was via blocking

LVDCCs-mediated Ca^{2+} influx, firstly we used whole-cell patch-clamp technology to test its effect on LVDCCs. The currents were recorded when ASMCs were depolarized from -70 mV to +40 mV without benidipine or nifedipine (positive control) (Fig. 2B left). While the currents were blocked in presence of benidipine or nifedipine (Fig. 2B right). Then we tested benidipine effect on Ca^{2+} influx activated by high K^+. Under Ca^{2+}-free condition, high K^+ could not induce ASM contraction, because Ca^{2+} influx didn't happen. But when the extracellular Ca^{2+} concentration restored to 2mM, ASM contracted immediately within high K^+, because Ca^{2+} influx occur (Fig. 3A). Following benidipine addition, high K^+-induced contraction was inhibited (Fig. 3A). In addition, high K^+ lost the ability to stimulate contraction on benidipine-preincubated ASM no matter whether extracellular Ca^{2+} concentration was 0 or 2 mM (Fig. 3B). Taken together, benidipine relaxation effect may be through blocking LVDCCs-mediated Ca^{2+} influx.

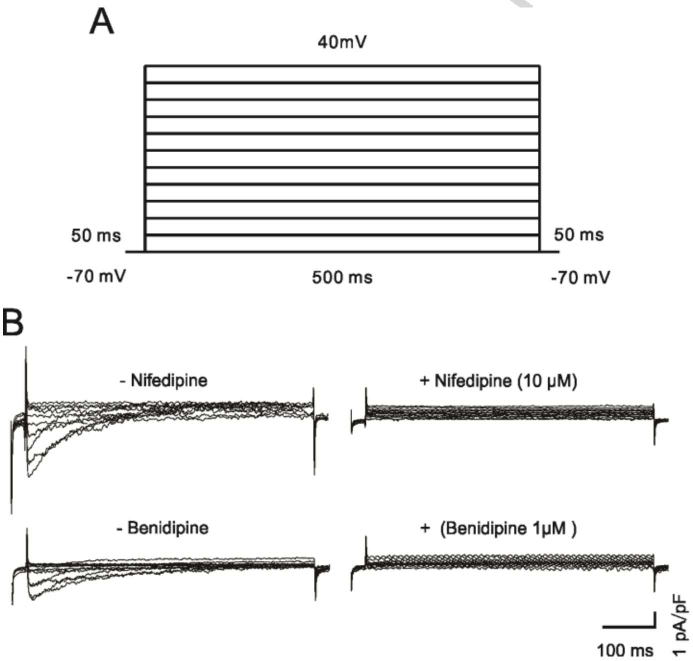

Fig. 2. Benidipine blocked LVDCCs currents on ASMCs. (A) LVDCCs currents record protocol; (B) LVDCCs currents record according to the protocol as figure A, both benidipine and nifedipine could block LVDCCs currents (n = 7); (C) *I-V* curve based on figure B.

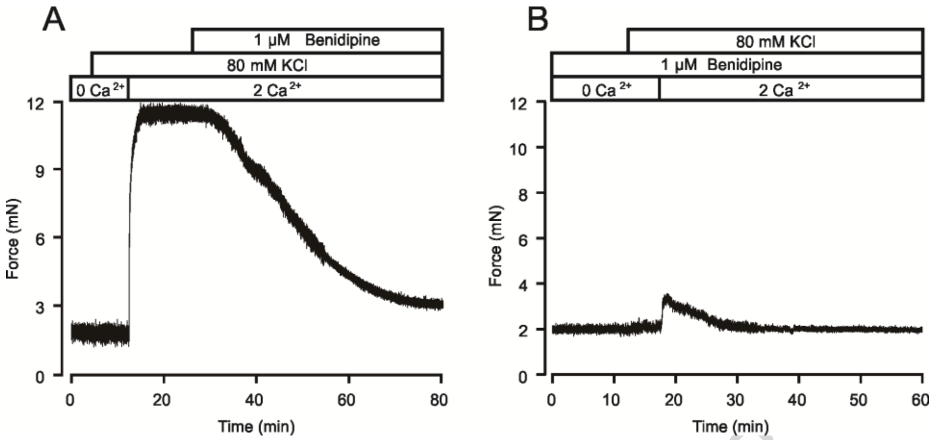

Fig. 3. Benidipine blocked high K^+-induced Ca^{2+} influx. (A) High K^+-induced contraction depended on extracellular Ca^{2+} influx (n = 7); (B) High K^+ lost the ability to stimulate contraction on benidipine-preincubated ASM with extracellular Ca^{2+} (n = 6).

3.3 Benidipine relaxed ACh-induced precontraction on mouse ASM

ACh is a kind of neurotransmitter, which is released by nerve cells in the body of human and many kinds of animals, functions as a signal to mediate other cells behaviors (such as muscle cells contraction and relaxation) [25, 26]. Here we used ACh-induced ASM contraction in vitro to imitate ASM contraction in vivo, and evaluated probable roles of benidipine on ASM under physiological conditions. As shown in Fig. 4, benidipine completely relaxed ACh-induced precontraction in a dose-dependent manner vs. vehicle, its maximal relaxation rate was 99.23 ± 1.21% and IC_{50} was 44.94 ± 1.62 μM based on the dose-effect curve.

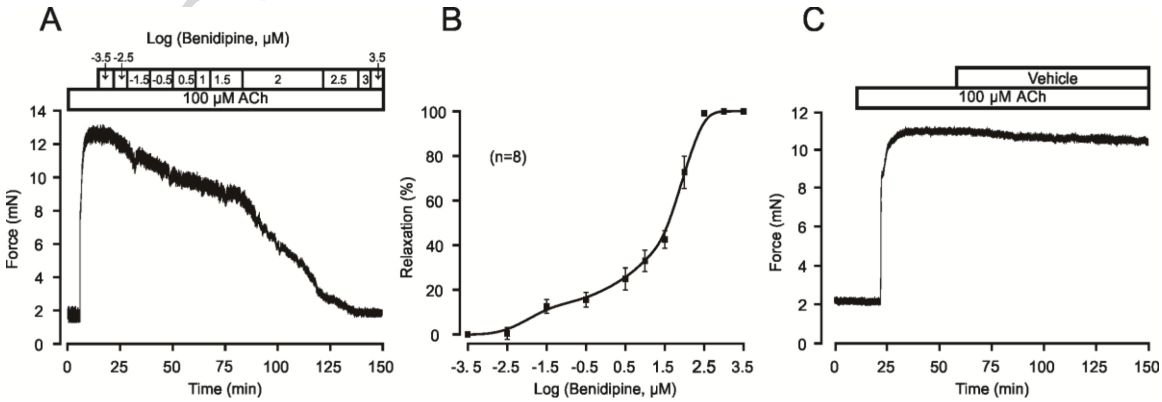

Fig. 4. Benidipine relaxed ACh-induced precontraction on mouse ASM. (A) Benidipine caused dose-dependent relaxations on high ACh-induced precontraction; (B) Relaxation effect curve of

benidipine (n = 8); (C) Vehicle (DMSO) had no relaxant effect on ACh-induced precontraction (n = 6).

It is known that both LVDCCs and NSCCs participate in ACh-induced ASM contractions [27, 28]. Benidipine blockage effect on LVDCCs had already been confirmed (Fig. 2), so we wondered what effects did benidipine have on non-LVDCCs (such as NSCCs). Nifedipine was used to excluding LVDCCs effect from ACh-induced precontraction. As shown in Fig. 5, 10 μM nifedipine partly inhibited ACh-induced precontraction，the relaxation rate was about 28%, the rest precontraction was fully inhibited by 316 μM benidipine (Fig. 5A), and vehicle had no effect on the rest precontraction (Fig. 5B). When ASM preincubated with nifedipine, ACh could also cause a dramatic contraction, which was suppressed by benidipine in a dose-dependent manner (Fig. 5C), the maximal relaxation rate was 93.43 ± 0.57％ and IC_{50} was 3.10 ± 0.07 μM based on the dose-effect curve (Fig. 5D). These results indicated that benidipine could completely relax ACh-induced precontraction, both LVDCCs and non-LVDCCs participated in the process. Considering that high concentration of benidipine may be poisonous to ASM tissues, we chose 100 μM for the following ACh associated experiments.

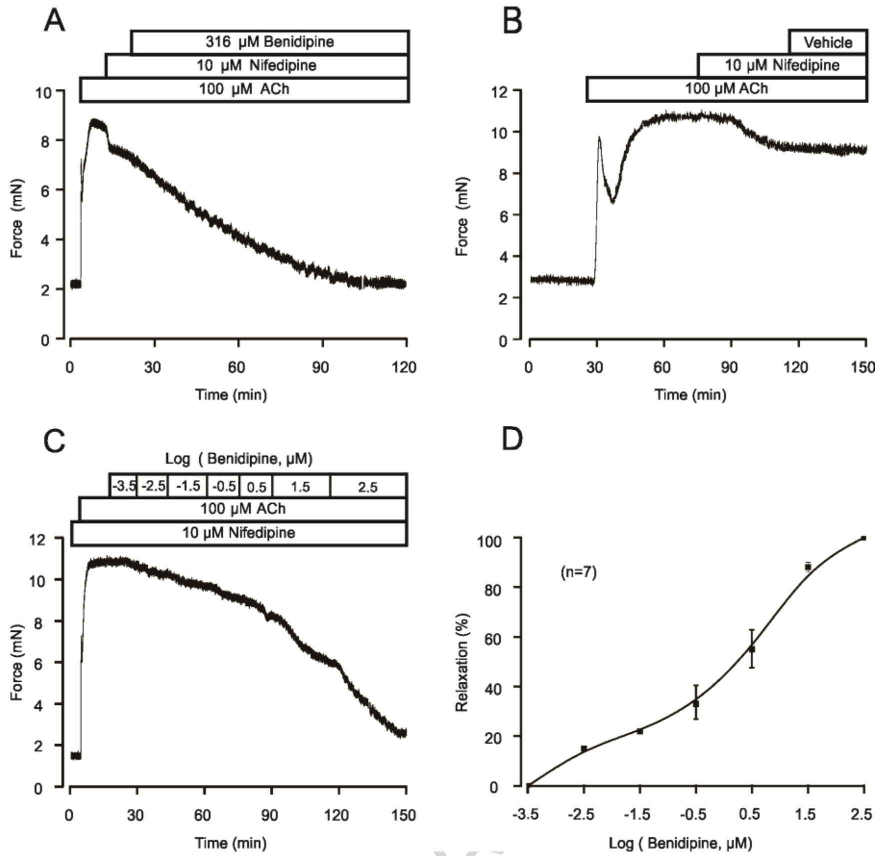

Fig. 5. Benidipine relaxed ACh-induced precontraction without LVDCCs effect. (A) Nifedipine partly inhibited ACh-induced precontraction, the rest precontraction was fully inhibited by 316 μM benidipine (n = 6); (B) Vehicle (DMSO) had no relaxant effect under same conditions as figure A (n = 6); (C) Removal effect of LVDCCs by adding nifedipine, benidipine also caused dose-dependent relaxations on high ACh-induced precontraction; (D) Dose-effect curve based on figure C (n = 7).

3.4 Benidipine blocked ACh-activated NSCCs currents and Ca^{2+} influx

Both LVDCCs- and NSCCs-mediated Ca^{2+} influxes contribute to ACh-induced ASM contraction. So it's interesting to elucidate whether benidipine relaxation effect was via blocking NSCCs-mediated Ca^{2+} influx. Whole-cell patch-clamp recordings were performed under ramp protocol from -80 mV to +60 mV (Fig. 6A). LVDCCs, Cl^- and K^+ currents were blocked by 10 μM nifedipine, 100 μM NA and 10 mM TEA respectively. ACh-activated NSCCs currents were obviously shown at -70 mV, while dramatically blocked by 100 μM benidipine (Fig. 6B).

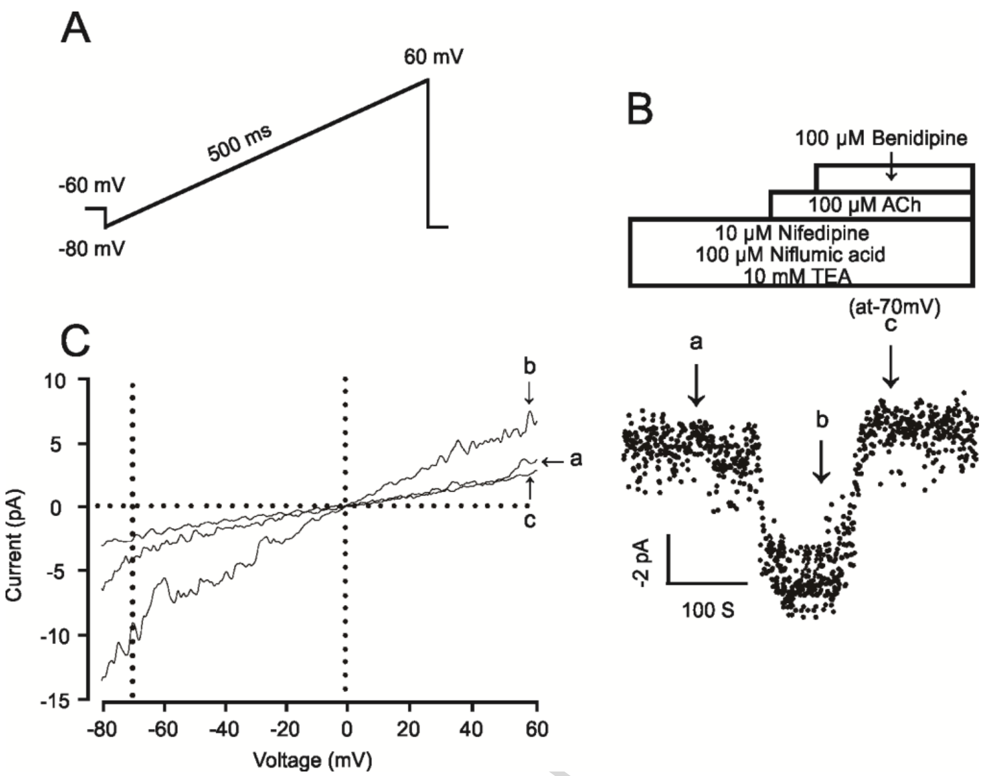

Fig. 6. Benidipine blocked ACh-activated NSCCs currents on ASMCs. (A) NSCCs currents record protocol; (B) *I-t* relationship was plotted at -70 mV; (C) The slope currents at times a, b, and c in figure B.

The effects of benidipine on ACh-activated Ca^{2+} influx were explored. Under Ca^{2+}-free condition, ACh could induce a sharp instantaneous contraction, probably because of intracellular Ca^{2+} transient release from sarcoplasmic reticulum. Subsequent addition of 2 mM Ca^{2+}, a bigger sharp contraction followed by a sustained contraction were shown in Fig. 7A. Benidipine inhibited the sustained contraction (Fig. 7B). Preincubation with benidipine and without Ca^{2+}, similar but smaller sharp instantaneous contractions occurred when ACh and 2 mM Ca^{2+} added, while the sustained contraction did not happen (Fig. 7C). In presence of nifedipine, benidipine also showed inhibition effects on ACh-induced contraction, probably through inhibition of NSCCs-mediated Ca^{2+} influx (Fig. 7D).

TRPCs, especially TRPC3, are important molecular components of native

NSCCs in ASMCs [29]. TRPC-encoded NSCCs cause Ca^{2+} influx in freshly isolated ASMCs during muscarinic stimulation [30]. To figure out whether or not benidipine inhibited ACh-stimulated Ca^{2+} influx through TRPC-encoded NSCCs, Pyr3 (selective TRPC3 inhibitor) and gadolinium (non-selective TRPC inhibitor) were used. In presence of nifedipine, the ACh-stimulated contraction was partially suppressed by Pyr3 (30 μM) and gadolinium (30 μM) due to Ca^{2+} influx inhibition, the relaxation rate were 22% and 18% respectively (Fig. 7E), and the rest contraction was inhibited by benidipine (Fig. 7F). Thus, benidipine blocked both TRPC-encoded and non-TRPC-encoded NSCCs-mediated Ca^{2+} influx.

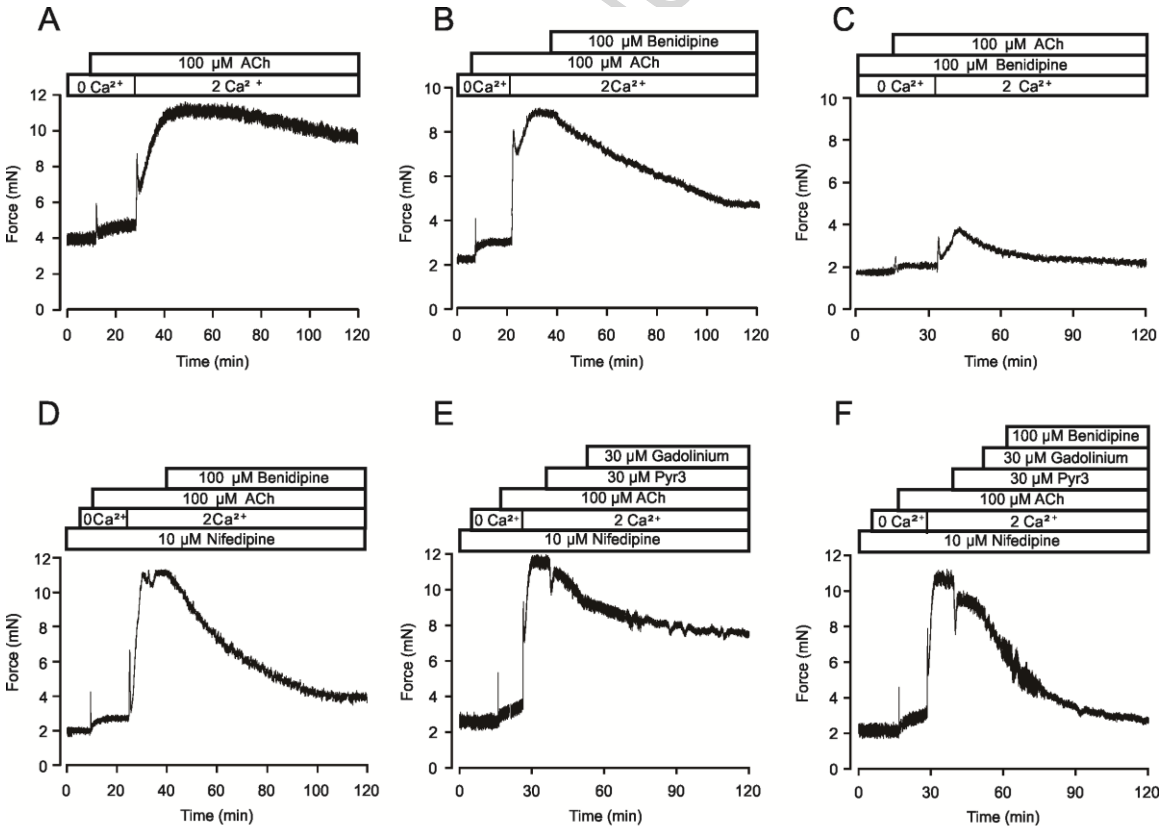

Fig. 7. Benidipine blocked ACh-activated Ca^{2+} influx. (A) ACh-activated sustained contraction depended on extracellular Ca^{2+} influx (n = 7); (B) Effect of benidipine on ACh-activated contraction under same conditions as figure A (n = 6); (C) Preincubation with benidipine, the sustained contraction stimulated by ACh did not happen (n = 6); (D) Benidipine blocked ACh-activated Ca^{2+} influx without LVDCCs effect (n = 6); (E) In presence of nifedipine, the

ACh-stimulated contraction was partially suppressed by Pyr3 (30 μM) and gadolinium (30 μM) respectively; (F) Benidipine inhibited the rest contraction under same conditions as figure E.

3.5 Effects of benidipine on high K^+- or ACh-induced precontraction after withdrawal

To investigate the dissociation characteristic of benidipine from Ca^{2+} channels, we test benidipine relaxation effect on mouse ASM after withdrawal. Fig. 8A showed that benidipine relaxation effect on high K^+-induced precontraction was not reversed after withdrawal. Same protocol was used in ACh-induced precontraction, a small part of benidipine relaxation effect was reversed after withdrawal (Fig. 8B). Hence, benidipine dissociated slowly from Ca^{2+} channels binding site in mouse ASM, its relaxation effect persisted for at least 40 mins after withdrawal (data not shown).

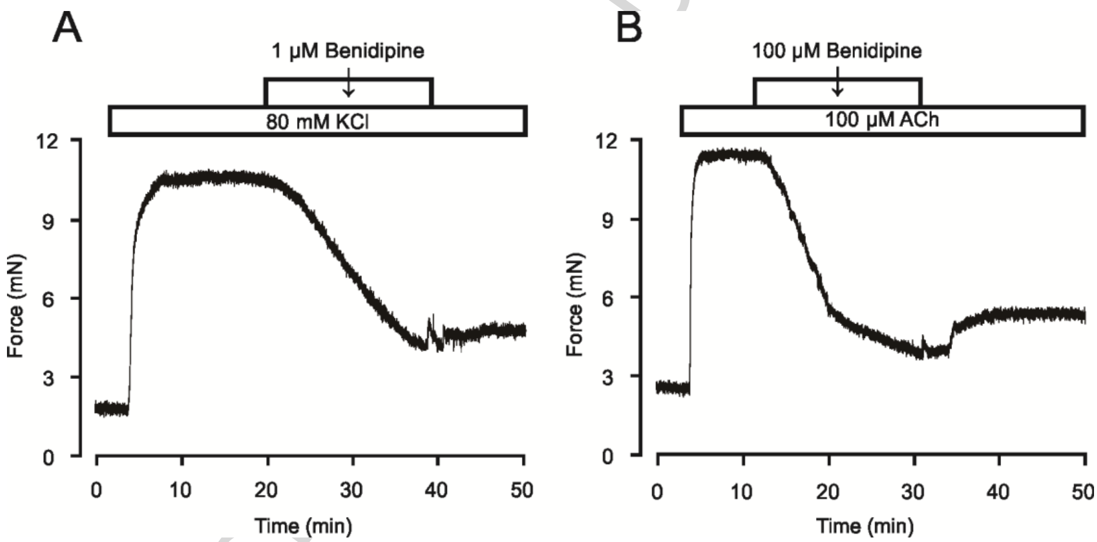

Fig. 8. Effects of benidipine on high K^+- or ACh-induced precontraction after withdrawal. (A) Benidipine relaxation effect on high K^+-induced precontraction was not reversed after withdrawal. (B) A small part of benidipine relaxation effect on ACh-induced precontraction was reversed after withdrawal.

4. Discussion

Airway hyperresponsiveness is one of the typical pathological features of asthma, which is thought to be caused by hypercontractility of ASM [31, 32]. ASM contraction depends on intracellular Ca^{2+} concentration, which is controlled by multiple Ca^{2+} channels, such as LVDCCs, NSCCs and TRPCs [33]. Therefore, screening Ca^{2+} channel antagonists and developing drugs to relax ASM will help to reduce airway hyperresponsiveness and even relieve asthma symptoms.

Benidipine is a long-acting triple Ca^{2+} channels (L, N, and T) blocker, used to treat hypertensionin in clinic, and shows renoprotective, cardioprotective and vascular endothelial protective effects [14]. The mainly pharmacological mechanism of antihypertension effect of benidipine is that it could relax VSM and myocardium through blocking LVDCCs [34-37]. In current study, Ca^{2+} antagonistic effect of benidipine in ASM was investigated for the first time, in which the contractile responses to high K^+ or ACh were taken as indicators.

First we investigated benidipine effects on high K^+-induced precontraction and LVDCCs currents in ASM. It has previously been reported that benidipine relaxed the contraction and inhibited ^{45}Ca-uptake induced by high K^+ (55 mM) in canine coronary artery [34]. And the relaxation activity of benidipine is of about the same degree as nifedipine in rabbit mesenteric artery [38]. Both benidipine and nifedipine block Ca^{2+} current which are activated by depolarization in guinea-pig cardiac ventricular cells through whole-cell patch clamp technique [35]. In this study, benidipine showed dose-dependent inhibition of high K^+ (80 mM) induced precontraction in mouse ASM (IC_{50}=0.16 μM) (Fig. 1), which relied on inhibition of extracellular Ca^{2+} influx (Fig. 3), and benidipine totally blocked LVDCCs currents when the holding potential was stepped from -70 mV to +40 mV at a concentration of 1μM (Fig. 2), while 10 μM nifedipine was used as control.

Then we investigated benidipine relaxation effects on ACh-induced precontraction and explored which Ca^{2+} channels were involved. Although high K^+ induced

LVDCCs-mediated Ca^{2+} influx is sufficient to produce a contraction, but this contraction is generally only a fraction of that activated by physiological agonists [33]. It means that there must be other Ca^{2+} channels which participate in intracellular Ca^{2+} increase evoked by physiological agonists. Several studies have reported that muscarinic agonists ACh and MCh activate NSCCs in freshly isolated ASMCs, which are always accompanied by a sustained intracellular Ca^{2+} increase due to extracellular Ca^{2+} influx [28, 30, 39]. Based on the molecular biological and electrophysiological experiments, TRPC family are considered to be the candidate molecules for NSCCs [40-42]. TRPC3 gene silencing inhibits ACh- and MCh-induced intracellular Ca^{2+} increase in ASMCs [30, 43]. So TRPC3 may be the major molecular component of NSCCs to contribute to muscarinic agonists-evoked Ca^{2+} influx in ASMCs.

Our study reveals that ACh-activated precontraction of ASM was partially affected by the LVDCCs blocker nifedipine (Fig. 5A, B), which is consistent with previous reports [44-46]. Benidipine caused dose-dependent relaxations on the ACh-induced precontraction with or without nifedipine (Fig. 4A, 5C), those effects also relied on the blockage of extracellular Ca^{2+} influx (Fig. 7B, D). These data indicate that benidipine did not only block LVDCCs but also other ion channels which allow Ca^{2+} influx. Electrophysiological results suggested that when LVDCC, Cl^- and K^+ currents were excluded, benidipine could block ACh-stimulated NSCCs currents (Fig. 6B), which were recorded at -70 mV by the voltage ramp protocol in single ASMCs (Fig. 6C). In presence of nifedipine, the ACh-stimulated contraction was partially inhibited by Pyr3 and gadolinium in succession for approximately 22% and 18% relaxation respectively when extracellular Ca^{2+} existed (Fig. 7E), then the rest contraction was mostly abolished by benidipine (Fig. 7F). So Ca^{2+} influx in ACh-stimulated contraction was not only mediated by TRPC3- or even other TRPC-encoded NSCCs, but also non-TRPC-encoded NSCCs, whatever benidipine could block all of them. However, the molecular mechanism of benidipine blocking NSCCs needs to be investigated in the future.

We also investigated benidipine relaxation effects on histamine-induced precontraction. Histamine, as an inflammatory stimulant, was used in vitro to imitate physiological conditions of ASM in vivo. In the presence of 31.6 μM histamine, benidipine partly relaxed the precontraction in a dose-dependent manner, the maximal relaxation rate was 67.8 ± 1.4% (Fig. S1). Histamine induces ASM contraction through H1 receptor coupling that triggers release of internal Ca^{2+} stores, followed by extracellular Ca^{2+} influx involving NSCCs, NCXs and LVDCCs activation [47, 48]. Which specific Ca^{2+} channels are affected by benidipine in histamine-induced contraction still need further investigation.

Usual Ca^{2+} antagonists, such as nifedipine, combine with DHP binding site directly, thereby inhibiting Ca^{2+} influx. While benidipine combinates with DHP binding site either directly or indirectly [14]. It is speculated that benidipine may have an additional interaction with cell lipid membrane or Ca^{2+} channel protein. That's why it cannot be washed away even after dissociation from DHP binding site in ventricular cells [35]. Our results also indicate that benidipine relaxation effects on high K^+- or ACh-induced precontraction were hard to reverse after withdrawal (Fig. 8).

5. Conclusion

Our research verified that benidipine, an antihypertension drug, could relax high K^+- or ACh-induced precontraction through blocking LVDCCs and NSCCs (including TRPCs) mediated Ca^{2+} influx in mouse ASM. This research represented benidipine with a new potential medicinal value for airway hyperresponsiveness.

Conflict of interest

The authors declare that there are no conflicts of interest with this study.

Acknowledgments

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References

[1] GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 388 (2016) 1545-1602.

[2] Global Burden of Disease Study 2013 Collaborators, Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet. 386 (2015) 743-800.

[3] GBD 2015 Mortality and Causes of Death Collaborators, Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 388 (2016) 1459-1544.

[4] T. Ueno-Iio, M. Shibakura, K. Iio, Y. Tanimoto, A. Kanehiro, M. Tanimoto, M. Kataoka, Effect of fudosteine, a cysteine derivative, on airway hyperresponsiveness, inflammation, and remodeling in a murine model of asthma. Life Sci. 92 (2013) 1015-1023.

[5] P.B. Noble, C.D. Pascoe, B. Lan, S. Ito, L.E. Kistemaker, A.L. Tatler, T. Pera, B.S. Brook, R. Gosens, A.R. West, Airway smooth muscle in asthma: linking contraction and mechanotransduction to disease pathogenesis and remodelling. Pulm Pharmacol Ther. 29 (2014) 96-107.

[6] M.J. Abramson, J. Walters, E.H. Walters, Adverse effects of beta-agonists: are they clinically relevant? Am J Respir Med. 2 (2003) 287-297.

[7] R.F. Coburn, C.B. Baron, Coupling mechanisms in airway smooth muscle. Am J Physiol. 258 (1990) L119-133.

[8] X.C. Liu, Q. Wang, Y.S. She, S. Chen, X. Luo, H. Xu, D.A. Zang, W.J. Zhang, J.Y. Qiu, B.B. Liu, J. Shen, Y.B. Peng, P. Zhao, L. Xue, W. Chen, L.Q. Ma, X. Fu, J. Chen, Q.H. Liu, M.F. Yu, Hypertonic saline inhibits airway smooth muscle contraction by inhibiting Ca(2+) sensitization. Clin Exp Pharmacol Physiol. 44 (2017) 1053-1059.

[9] Y. Chiba, M. Misawa, Probable involvement of the augmented agonist-induced Ca2+ sensitization of airway smooth muscle contraction in the pathogenesis of airway hyperresponsiveness. Nihon Yakurigaku Zasshi. 114 (1999) 185-190.

[10] H. Kosaka, K. Hirayama, N. Yoda, K. Sasaki, T. Kitayama, H. Kusaka, M. Matsubara, The L-, N-, and T-type triple calcium channel blocker benidipine acts as an antagonist of mineralocorticoid receptor, a member of nuclear receptor family. Eur J Pharmacol. 635 (2010) 49-55.

[11] A. Inayoshi, Y. Sugimoto, J. Funahashi, S. Takahashi, M. Matsubara, H. Kusaka, Mechanism underlying the block of human Cav3.2 T-type Ca2+ channels by benidipine, a dihydropyridine Ca2+ channel blocker. Life Sci. 88 (2011) 898-907.

[12] H. Kobayashi, S. Kobayashi, Relationship between plasma concentration and antihypertensive effect of the dihydropyridine calcium antagonist, benidipine, in rats. J Pharm Pharmacol. 49 (1997) 1200-1204.

[13] W. Qi, W. Fan, R.S.S. investigators, Antihypertensive efficacy and safety of benidipine and its effects on cardiac structure and function in elderly Chinese patients with mild to moderate hypertension: an open-label, long-term study. Arzneimittelforschung. 61 (2011) 160-166.

[14] K. Yao, K. Nagashima, H. Miki, Pharmacological, pharmacokinetic, and clinical properties of benidipine hydrochloride, a novel, long-acting calcium channel blocker. J Pharmacol Sci. 100 (2006) 243-261.

[15] E. Arakawa, K. Hasegawa, Benidipine, a calcium channel blocker, regulates proliferation and phenotype of vascular smooth muscle cells. J Pharmacol Sci. 100 (2006) 149-156.

[16] T. Moriyama, A. Karasawa, Cardiovascular effects of benidipine and amlodipine in isolated tissues and anesthetized dogs. Biol Pharm Bull. 17 (1994) 1468-1471.

[17] T. Zhang, X.J. Luo, W.B. Sai, M.F. Yu, W.E. Li, Y.F. Ma, W. Chen, K. Zhai, G. Qin, D. Guo, Y.M. Zheng, Y.X. Wang, J.H. Shen, G. Ji, Q.H. Liu, Non-selective cation channels mediate chloroquine-induced relaxation in precontracted mouse airway smooth muscle. PLoS One. 9 (2014) e101578.

[18] H. Tan, J. Lei, L. Xue, C. Cai, Q.H. Liu, J. Shen, Relaxing Effect of TSU-68, an Antiangiogenic Agent, on Mouse Airway Smooth Muscle. Cell Physiol Biochem. 41 (2017) 2350-2362.

[19] J. Huang, L.Q. Ma, Y. Yang, N. Wen, W. Zhou, C. Cai, Q.H. Liu, J. Shen, Chloroform Extract of Artemisia annua L. Relaxes Mouse Airway Smooth Muscle. Evid Based Complement Alternat Med. 2017 (2017) 9870414.

[20] X. Yang, M.F. Yu, J. Lei, Y.B. Peng, P. Zhao, L. Xue, W. Chen, L.Q. Ma, Q.H. Liu, J. Shen, Nuciferine Relaxes Tracheal Rings via the Blockade of VDLCC and NSCC Channels. Planta Med. 84 (2018) 83-90.

[21] H. Karaki, N. Urakawa, P. Kutsky, Potassium-induced contraction in smooth muscle. Nihon Heikatsukin Gakkai Zasshi. 20 (1984) 427-444.

[22] G.B. Weiss, Calcium and contractility in vascular smooth muscle, in: T. Narahashi, C.P. Bianchi (Eds.), Advances in general and cellular pharmacology, Plenum Press, New York, NY, USA, 1977, pp.

71-154.
[23] G.B. Weiss, Stimulation with high potassium, in: E.E. Daniel, D.M. Paton (Eds.), Smooth Muscle, Plenum Press, New York, NY, USA, 1975, pp. 339-345.
[24] Y. Cai, Y. Lei, J.G. Chen, L. Cao, X.D. Yang, K.H. Zhang, Y.X. Cao, Erythromycin relaxes BALB/c mouse airway smooth muscle. Life Sci. 221 (2019) 135-142.
[25] P. Tiwari, S. Dwivedi, M.P. Singh, R. Mishra, A. Chandy, Basic and modern concepts on cholinergic receptor: A review. Asian Pacific Journal of Tropical Disease. 3 (2013) 413-420.
[26] L.E. Kistemaker, T.A. Oenema, H. Meurs, R. Gosens, Regulation of airway inflammation and remodeling by muscarinic receptors: perspectives on anticholinergic therapy in asthma and COPD. Life Sci. 91 (2012) 1126-1133.
[27] Y.X. Wang, B.K. Fleischmann, M.I. Kotlikoff, M2 receptor activation of nonselective cation channels in smooth muscle cells: calcium and Gi/G(o) requirements. Am J Physiol. 273 (1997) C500-508.
[28] B.K. Fleischmann, Y.X. Wang, M.I. Kotlikoff, Muscarinic activation and calcium permeation of nonselective cation currents in airway myocytes. Am J Physiol. 272 (1997) C341-349.
[29] P.B. Helli, L.J. Janssen, Properties of a store-operated nonselective cation channel in airway smooth muscle. Eur Respir J. 32 (2008) 1529-1539.
[30] Y.X. Wang, Y.M. Zheng, Molecular expression and functional role of canonical transient receptor potential channels in airway smooth muscle cells. Adv Exp Med Biol. 704 (2011) 731-747.
[31] A.M. Lauzon, J.G. Martin, Airway hyperresponsiveness; smooth muscle as the principal actor. F1000Res. 5 (2016).
[32] W.K. Jung, D.Y. Lee, Y.H. Choi, S.S. Yea, I. Choi, S.G. Park, S.K. Seo, S.W. Lee, C.M. Lee, S.K. Kim, Y.J. Jeon, I.W. Choi, Caffeic acid phenethyl ester attenuates allergic airway inflammation and hyperresponsiveness in murine model of ovalbumin-induced asthma. Life Sci. 82 (2008) 797-805.
[33] L.J. Janssen, Ionic mechanisms and Ca(2+) regulation in airway smooth muscle contraction: do the data contradict dogma? Am J Physiol Lung Cell Mol Physiol. 282 (2002) L1161-1178.
[34] A. Karasawa, K. Kubo, Calcium antagonistic effects and the in vitro duration of actions of KW-3049, a new 1,4-dihydropyridine derivative, in isolated canine coronary arteries. Jpn J Pharmacol. 47 (1988) 35-44.
[35] M. Yamamoto, Y. Gotoh, Y. Imaizumi, M. Watanabe, Mechanisms of long-lasting effects of benidipine on Ca current in guinea-pig ventricular cells. Br J Pharmacol. 100 (1990) 669-676.
[36] Y. Gotoh, K. Muraki, Y. Imaizumi, M. Watanabe, Effects of benidipine, an 1,4-dihydropyridine derivative, on single L-type Ca channel currents in vascular smooth muscle cells. Clinical Report. 26 (1992) 3897-3901.
[37] S. Yamada, M. Nakajima, T. Kusaka, S. Uchida, R. Kimura, In vivo receptor binding of benidipine and amlodipine in mesenteric arteries and other tissues of spontaneously hypertensive rats. Life Sci. 70 (2002) 1999-2011.
[38] A. Karasawa, K. Kubo, K. Shuto, T. Oka, N. Nakamizo, Pharmacological actions of benidipine hydrochloride in several isolated smooth muscles and myocardium. Arzneimittelforschung. 38 (1988) 1722-1730.
[39] T. Yamashita, S. Kokubun, Nonselective cationic currents activated by acetylcholine in swine tracheal smooth muscle cells. Can J Physiol Pharmacol. 77 (1999) 796-805.
[40] I. So, K.W. Kim, Nonselective cation channels activated by the stimulation of muscarinic receptors in mammalian gastric smooth muscle. J Smooth Muscle Res. 39 (2003) 231-247.

[41] Y. Takai, R. Sugawara, H. Ohinata, A. Takai, Two types of non-selective cation channel opened by muscarinic stimulation with carbachol in bovine ciliary muscle cells. J Physiol. 559 (2004) 899-922.

[42] R. Sugawara, Y. Takai, M. Miyazu, H. Ohinata, A. Yoshida, A. Takai, Agonist and antagonist sensitivity of non-selective cation channel currents evoked by muscarinic receptor stimulation in bovine ciliary muscle cells. Auton Autacoid Pharmacol. 26 (2006) 285-292.

[43] T.A. White, A. Xue, E.N. Chini, M. Thompson, G.C. Sieck, M.E. Wylam, Role of transient receptor potential C3 in TNF-alpha-enhanced calcium influx in human airway myocytes. Am J Respir Cell Mol Biol. 35 (2006) 243-251.

[44] J.M. Farley, P.R. Miles, The sources of calcium for acetylcholine-induced contractions of dog tracheal smooth muscle. J Pharmacol Exp Ther. 207 (1978) 340-346.

[45] J.P. Bourreau, A.P. Abela, C.Y. Kwan, E.E. Daniel, Acetylcholine Ca2+ stores refilling directly involves a dihydropyridine-sensitive channel in dog trachea. Am J Physiol. 261 (1991) C497-505.

[46] E.E. Daniel, J. Jury, R. Serio, L.P. Jager, Role of depolarization and calcium in contractions of canine trachealis from endogenous or exogenous acetylcholine. Can J Physiol Pharmacol. 69 (1991) 518-525.

[47] M.I. Kotlikoff, R.K. Murray, E.E. Reynolds, Histamine-induced calcium release and phorbol antagonism in cultured airway smooth muscle cells. Am J Physiol. 253 (1987) C561-566.

[48] P. Algara-Suarez, C. Romero-Mendez, T. Chrones, S. Sanchez-Armass, U. Meza, S.M. Sims, R. Espinosa-Tanguma, Functional coupling between the Na+/Ca2+ exchanger and nonselective cation channels during histamine stimulation in guinea pig tracheal smooth muscle. Am J Physiol Lung Cell Mol Physiol. 293 (2007) L191-198.