

Dandelion Extract Relaxes Mouse Airway Smooth Muscle via the Blockade of VDCC and NSCC Channels

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Abstract

Background: Asthma is one of the intractable diseases recognized by the world medical community. The current widely used bronchodilators β_2 -adrenal receptor agonists for asthma have limited therapeutic effects, which make it necessary to develop novel drugs with increased efficacy but fewer side-effects for antiasthma treatments. In this study, we attempted to investigate the relaxing effects and mechanism of an ethyl acetate extract from dandelion (EAED) on mouse airway smooth muscle (ASM).

Methods: The effects of EAED on the agonist-induced precontraction in mouse ASM were evaluated with force measurement. Mouse lung slices were conducted to study the effect of EAED on bronchial ASM. Intracellular Ca^{2+} concentration was measured using calcium imaging system. VDLCCs and NSCCs currents was measured by patch. Lung function of groups of healthy or asthmatic mice was assessed by forced oscillation technique (FOT).

Results: EAED inhibits ACH-induced sustained contractions of the whole ASM by inhibiting VDLCC, NSCC and some unknown channels, decreases agonist-induced increasing cytosolic free Ca^{2+} concentration in ASMCS, blocks VDLCCs and NSCCs currents and relieve respiratory resistance of healthy and asthmatic mice.

Conclusions: EAED may have potential beneficial effects on improving asthma attacks.

Background

Asthma, as a major chronic respiratory disease, threatens the health of hundreds of millions of people around the world ¹. The rate of consultation and mortality of asthma patients has increased year by year ². Airway inflammation, airway hyperresponsiveness and airway remodeling are important pathophysiological characteristics of asthma. ASM is the key tissue regulating airway resistance, hyperreactivity and contraction, remarkable features of asthma ³. Airway smooth muscle cells (ASMCs) are one important cell type in the ASM and excessive contraction of ASMCs lead to the occurrence of symptoms of asthma by narrowing the airway lumen and limiting gas exchange ³. ASM contraction induced by agonists (*i.e.* acetylcholine, 5-hydroxytryptamine) usually relies on an increase in $[\text{Ca}^{2+}]_i$ and composes of Ca^{2+} oscillations. These Ca^{2+} oscillations are caused by the release of Ca^{2+} from the intracellular calcium pool and the influx of Ca^{2+} from the extracellular ⁴.

At present, the first-line treatment for asthma is still β_2 adrenergic receptor (β_2 -AR) agonists combined with glucocorticoids. However, this therapeutic strategy can cause multiple severe side-effects, such as headache, tremors, palpitations and cardiac failure. Thus, in this study, we attempted to develop a safe and effective drug from natural plant to inhibit ASM contraction.

Dandelion is a perennial herbaceous plant with the scientific name *Taraxacum mongolicum* Hand.-Mazz. (TMHM), belonging to the composite family. Its main chemical components are taraxasterol, choline,

organic acid and inulin and other healthy nutrients⁵, thus it is often eaten as a nutritious wild vegetable. In addition, it also has many pharmacological effects. Modern pharmacological studies show that dandelion has the functions of antibacterial^{6,7}, antiviral⁸, anticancer⁹⁻¹³, antioxidant¹⁴, anti-inflammatory¹⁵⁻¹⁷, anti-allergic and so on. In terms of alleviating airway inflammation, one distinctive feature of asthma, it has been reported that the organic acid components of TMHM (TMHM-OAC) can improve lipopolysaccharide (LPS)-induced histopathological damage of tracheal tissues¹⁸ and reduce LPS-induced inflammation in normal human bronchial epithelial (NHBE) cells¹⁹, which could be beneficial for the treatment of acute tracheobronchitis. Taraxasterol was also found to be effective in improving ovalbumin (OVA)-induced allergic asthma in mice²⁰. There is also a lot of literature on the potential efficacy of dandelion in mice. However, whether a certain component from Dandelion has a potential effect in inhibiting mouse ASM contraction has not been investigated.

In the present study, we found that EAED exerted inhibitory effects on mouse ASM precontraction, and the underlying mechanism was also investigated.

Methods

Dandelion Extraction

Dandelion was purchased from Beijing TongrenTang (Wuhan, China). The air-dried dandelion (0.5 Kg) was milled into powder and soaked in 80% ethanol (5L) for 3 days. Then the crude ethanol extract was obtained by filtration and rotary evaporation. The ethyl acetate extract of dandelion was obtained by phase separation extraction. The dried ethyl acetate extract of dandelion was dissolved in 3% DMSO for the experiments.

Reagents

Nifedipine, acetylcholine chloride (ACH), and pyrazole3 (Pyr3) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Fura-2 AM were purchased from Invitrogen (Eugene, OR, USA). Other chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Animals and Establishment of asthma model mice

Six-weeks-old male BALB/c mice were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China) and were housed in a specific pathogen free (SPF) grade animal facility. All animal experiments were performed in accordance with the requirements of the Institutional Animal Ethics Committee of the South-Central University for Nationalities. The license number is 2016-SCUEC-AEC-0030. Asthmatic mice were prepared as described previously²¹. And, the mice were sacrificed with an intraperitoneal injection of sodium pentobarbital (250 mg/kg, purity \geq 98%; Sigma) before or after performing each experiment.

Contraction measurement of tracheal and bronchial ASM

Mouse ASM tension was measured as previously described²². Briefly, mouse TRs were clipped clean, cut about 1 cm and hung on the triangular hook in a 6 mL PSS bubbled with 95% O₂ and 5% CO₂ at 37°C. A 300 mg preload is set. TRs was equilibrated for 1 hour and then prestimulated with 100 μM ACH or 80 mM KCl for 20 minutes. After resting for another 20 minutes, experiments were performed.

ASM force measurements in mouse lung slices were performed as previously described. In brief, the lung slices were cut and placed in a chamber perfused using Hanks' balanced salt solution (HBSS). The LSM 700 laser confocal microscope and Zen 2010 software (Carl Zeiss, Göttingen, Germany) were performed to measure the cross-sectional areas of the bronchial lumen. Each part of the experiment was independently repeated for more than six times (i.e., more than six random mice).

Measurement of plasma calcium concentration in ASMCs

Mouse acute detached ASMCs suspension was diluted to an appropriate density and was treated with poly-D-lysine. A specially designed cell bath of the passed slides is placed in an inverted microscope connected to the calcium imaging system. Poly-D-lysine could help cells adhere to the glass slide. Then cells were dyed with 2.5 M fura-2 AM color. After 20 minutes of staining, PSS physiological saline solution was perfusion for 5 minutes to wash away the excess fura-2. With calcium imaging TILL imaging system: 340 and 380 fluorescence images of the cell area, Ratio (340/380) can be used to reflect the intracellular calcium concentration. Each part of the experiment was based on more than 30 ASMCs (i.e., more than six random mice).

Patch

VDLCCs and NSCCs currents induced by ACH were recorded using EPC-10 patch clamp amplifier (HEKA, Lambrecht, Germany) and utilizing whole-cell recording mode. VDLCCs current was stimulated by step voltage from - 70 mV to + 40 mV. NSCCs current was recorded with nifedipine, niflumic acid and TEA in the solution in advance, under the condition of the ramp, voltage from - 80 mV to + 60 mV in 500 ms. Each part of the experiment was independently repeated for more than six times (i.e., more than six random ASMCs/mice).

Pulmonary function measurement

Lung function of groups of healthy or asthmatic mice were measured using forced oscillation technique (FOT). Mice were weighed and anesthetized with an injection of sodium pentobarbital (10 mg/kg, ip). After complete anesthesia, the mice were intubated and placed in a flow-type body plethysmograph and connected via the endotracheal cannula to a flexiVent system (SCIREQ Inc., Montreal, Canada). Lung function was assessed subsequently by FOT at baseline and following multiple concentrations of aerosolized ACH (3.125– 50 mg/mL) dissolved with vehicle or EAED. Respiratory system resistance (Rrs) were calculated in the flexiVent software to reflect the degree of airway hyperresponsiveness. Each part of the group experiment was independently repeated for more than six times (i.e., more than six random mice).

Data analysis

The results are expressed as mean \pm SEM. Comparisons between two groups were performed with Student's t-test using Origin 9.0 software (OriginLab, Northampton, USA). Differences with $p \leq 0.05$ were considered significant.

Results

EAED inhibits TRs contraction

We first study the effect of EAED on TRs contraction. TRs were precontracted by 80mM KCl, then EAED was added when the contraction reaches a plateau. The contraction was inhibited in a dose-dependent manner (Figure 1A). As a comparison, vehicle (PSS containing 3% DMSO) which was used to dissolved EAED was added in the same doses upon the stabilized contraction (Figure 1B) and no relaxation emerging subsequently. This suggests that EAED is indeed acting as a role to relax airway smooth muscle. The values of half maximal inhibitory concentration (IC_{50}) of EAED was 0.063 ± 0.005 mg/mL (Figure 1C). It is also shown that contraction induced by 80 mM KCl was almost completely inhibited when the concentration of EAED reached 1 mg/mL. These results were from 7 TRs of 7 mice.

Figure 1. EAED inhibited high K^+ -induced tracheal ring contraction. (A) 80 mM K^+ induced a sustained contraction in a mouse TR, which was blocked by EAED in a concentration dependent way. The dose-inhibition curve is presented. (B) Similar experiments were performed, except that vehicle (PSS containing 3% DMSO) was added as control. (C) The dose-inhibition curve is presented. The IC_{50} of EAED was 0.063 ± 0.005 mg/mL. The data were obtained from 7 TRs.

Similarly, EAED was added after the contraction arising from 100 μ M ACH reached a peak and we could find a gradually obvious inhibition on the precontracted TRs (Figure 2A). At the same time, vehicle (PSS containing 3% DMSO) was also added in the same doses upon the steady contraction as a control (Figure 2B), which also exerted no relaxation. The dose-relaxation relationships were analyzed and the IC_{50} of EAED was 0.139 ± 0.04 mg/mL in this case (Figure 2C). We could also find the concentration of EAED inducing maximum relaxation was 3.16 mg/mL. These experiments above indicated that EAED could block high K^+ - and ACH-induced TRs precontraction. In addition, adding 3.16 mg/mL EAED without giving any agonist in advance exhibited a small contraction at once and returning to baseline later (Figure 2D), which indicated EAED had no effect on tracheal ring in resting state.

Figure 2. Contraction induced by ACH (100 μ M) was inhibited by cumulative addition of EAED. (A) Following addition of ACH, a TR reached a sustained contraction, which was inhibited following cumulative application of EAED. (B) Similar experiments were conducted, except that vehicle (PSS containing 3% DMSO) was added as control. (C) The summary results of EAED-induced relaxation in 7 TRs. The IC_{50} of EAED was 0.139 ± 0.04 mg/mL. (D) EAED had no effects on the basic tone of ASM.

EAED blocks bronchial smooth muscle contraction

To investigate whether EAED has a similar relaxant effect on mouse bronchial smooth muscle, we observed the effect of EAED on lung slices. After adding 100 μM ACH, the area of tracheal cavity decreased, but with the addition of EAED, the area of the lumen recovered (Figure 3A). The summary data are shown in Figure 3B from 6 lung slices of 5 mice. After adding 100 μM ACH for 40 minutes, the area of the lumen reduced to approximately 48%, which extended to about 82% compared to the initial value with subsequent application of 3.16 mg/mL EAED for 120 minutes. These results suggested that EAED may also inhibit the contraction of the bronchial smooth muscle.

Figure 3. EAED inhibits contraction in lung slices. (A) The airway lumen area in a lung slice was decreased by ACH and was markedly increased after the addition of EAED. (B) The summary results are shown. Data were from 6 lung slices of 5 mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

EAED exerts diastolic effects by inhibiting L-type Ca^{2+} , TRPC3, and/or STIM/Orai channels

To investigate the mechanism of the EAED inhibiting ACH-induced contraction, 10 μM nifedipine, a selective blocker of VDCCs, was added after contraction by ACH (Figure 4A). Then we could find the contractions were partial blocked. The relaxation value is about 18%. The remaining part were further blocked by EAED, relaxing to approximately 95% compared to baseline (Figure 4B). The above summary data were conducted from 7 TRs of 7 mice.

Then, we further investigate the nifedipine-resistant component of EAED-induced relaxation. Hence, we first incubated TRs with 10 μM nifedipine for 15 minutes, adding ACH subsequently, and we then observed the effect of Pyr3. The summary results from 6 TRs of 6 mice showed that Pyr3 induced partial relaxation, about 25% (Figure 4C), and the remaining contractions were completely blocked by EAED, almost 100% (Figure 4D).

Figure 4. Nifedipine, Pyr3 partially inhibits ACH-induced contraction, respectively. (A) ACH (100 μM) induced the contraction of mouse TRs, which was partially inhibited by 10 μM Nifedipine, and the rest was inhibited by 3.16 mg/mL EAED. (B) The summary results are shown from 7 TRs. (C) Mouse TRs were preincubated with 10 μM Nifedipine. ACH induced tracheal ring contraction, which was partially blocked by Pyr3, and the rest was completely inhibited by 3.16 mg/mL EAED. (D) The summary results are shown from 6 TRs.

EAED inhibit Ca^{2+} influx induced by high K^{+} and additional Ca^{2+} release induced by ACH

To further confirm the relationship between these channels and relaxation, the experiment of zero calcium and physiological calcium conversion was designed. The result in Figure 5A showed that when the

tracheal ring was at 0 Ca²⁺, high K⁺ still activated the VDLC channel but the intracellular Ca²⁺ concentration did not increase. So it could not cause tracheal ring contraction. When the extracellular [Ca²⁺]_i returned to 2 mM, the extracellular Ca²⁺ flowing rapidly, the intracellular [Ca²⁺]_i increased and the tracheal was constricted. This contraction could be inhibited by 1 mg/mL EAED. Furthermore, incubation with EAED almost completely abolished the contraction induced by 2 mM Ca²⁺ (Figure 5B). From these results, it could be concluded that EAED relaxing precontracted tracheal smooth muscle induced by high K⁺ was mediated by inhibition of VDLC channel mediating Ca²⁺ influx.

ACH can activate both VDLC and NSCC channels, which leads to extracellular Ca²⁺ influx, release of Ca²⁺ from the sarcoplasmic reticulum into the cytoplasm, increased Ca²⁺ concentration and finally causes contraction of tracheal smooth muscle. Under the condition of zero calcium, ACH was added. Since there was no Ca²⁺ outside the cell, it caused a transient release of Ca²⁺ in the sarcoplasmic reticulum, leading a transient of the contraction. When the extracellular [Ca²⁺]_i was restored to 2 mM, Ca²⁺ in cytoplasm was increased by the interaction of Ca²⁺ in sarcoplasmic reticulum and extracellular Ca²⁺ increasing (Figure 5C). So the trachea appeared a continuous and stable contraction and this contraction could be inhibited by 3.16 mg/mL EAED. Moreover, Under Ca²⁺-free conditions (0 Ca²⁺ and 0.5 mM EGTA) with conducted in the presence of EAED, ACH induced no transient contraction. Following the addition of 2 mM Ca²⁺, only a very weak contraction occurred, which was gradually back to baseline (Figure 5D). These results indicated that EAED-induced relaxation was through inhibiting ACH-elicited Ca²⁺ influx and Ca²⁺ release.

Figure 5. EAED blocks high K⁺-evoked Ca²⁺ influx and ACH-elicited Ca²⁺ influx and Ca²⁺ release. (A) A representative force tracing of 4 TRs. In the absence of calcium ions in the bath solution (0 Ca²⁺ and 0.5 mM EGTA), high K⁺ could not cause tracheal ring contraction. When the calcium ion concentration was restored to 2 mM, a sustained and stable contractile reaction was produced, which was inhibited by the subsequent addition of EAED. (B) Identical experiments were performed as described in the presence of 1mg/ml EAED, and high K⁺-induced contraction did not appear after the restoration of 2 mM Ca²⁺. (C) After blocking the VDLC channel with nifedipine, ACH was added in the bath solution without calcium, and the tracheal ring experienced an instantaneous internal calcium release process. After the calcium ions concentration recovered to 2 mM, the tracheal ring produced a stable contraction reaction, which could be completely inhibited by 3.16 mg/mL EAED added later to the baseline level. (D) After 3.16 mg/mL EAED pretreatment, the process of internal calcium release was significantly inhibited under the condition of 0 Ca²⁺ and 2 mM Ca²⁺ conversion experiment, and the contraction reaction caused by ACH was also significantly inhibited when the calcium ion concentration was restored to 2 mM.

EAED inhibits Ca²⁺ elevation in single ASMCS

Then, we attempted to observe the effect of EAED on intracellular Ca^{2+} in single ASMC conducted in the calcium imaging TILL system. The result showed that high K^+ (Figure 6A) and ACH-induced (Figure 6C) increases of intracellular Ca^{2+} were inhibited by 1 mg/mL or 3.16 mg/mL EAED. The values of Ratio (340/380) at the sites indicated by a, b and c were obtained and summary results from 35/30 cells of 5 mice were shown (Figure 6B and D). After high K^+ was added, the values of Ratio (340/380) increased from 0.51 ± 0.01 at point a to 0.75 ± 0.02 at point b, reducing to 0.35 ± 0.01 at point c with subsequent addition of 1 mg/mL EAED. Similar results were shown in the ACH-stimulated increasing $[\text{Ca}^{2+}]_i$, where the values of Ratio (340/380) increased from 0.44 ± 0.01 at point a to 0.55 ± 0.01 at point b, reducing to 0.33 ± 0.01 at point c with subsequent addition of 3.16 mg/mL EAED. It is suggested that the decreases of $[\text{Ca}^{2+}]_i$ were owing to inhibition of above Ca^{2+} permeant-ion channels by EAED.

Figure 6. EAED inhibits high K^+ and ACH-induced Ca^{2+} increases in single tracheal smooth muscle cells. (A) 80 mM K^+ induced a transient and a sustained increase of intracellular Ca^{2+} . The latter was inhibited following the addition of EAED. The values at the sites indicated by a, b and c were obtained. (B) Summary results from 35 cells of 5 mice. $***P < 0.001$. (C) The increase of calcium level in tracheal smooth muscle cells induced by ACH was inhibited by 3.16 mg/mL of EAED. (D) Summary results from 30 cells of 5 mice. $***P < 0.001$.

EAED effectively blocks VDLCCs and NSCCs currents

In order to further clarify the underlying mechanism, we measured the currents regulated by VDLCCs and NSCCs. As shown in figure 7A, the VDLCCs current was completely blocked by 10 μM nifedipine and 1 mg/mL EAED. The statistical data of 6 cases of cells in each of the two groups of experiments shows that at +10 mV, 1 mg/mL EAED and 10 μM nifedipine completely blocked the current.

To test whether EAED affects the opening of NSCCs channels, nifedipine, niflumic acid and TEA were added to exclude the influence of VDLCCs, K^+ and Cl^- currents, respectively. The results showed that NSCCs current could be blocked by 3.16 mg/mL EAED under -70 mV voltage conditions (Figure 7B). These results showed that EAED could completely inhibit the opening of NSCCs channel induced by ACH.

Figure 7. EAED blocks VDLCCs and NSCCs currents. (A) Protocol for measuring VDLCCs current of a single tracheal smooth muscle. (B) VDLCCs current was blocked by EAED or Nifedipine under the condition of depolarization of cell membrane. (C) The I-V curve was drawn based on the experimental results of six different tracheal smooth muscle cells. (D) Protocol for recording NSCCs currents in a single tracheal smooth muscle. (E) At -70mv, point a is the NSCCs channel state when K^+ , Cl^- and VDLCCs currents are excluded in the physiological state; Point b is the NSCCs channel opening after 100 μM ACH stimulation, reaching the plateau stage; Point c is the state of the NSCCs channel when 3.16 mg/mL EAED is added. (F) Broken line diagram obtained by experimental statistics of net slope current at three

time points a, b and c based on figure B of 6. (G) The average currents for time points b and c at -70 mV (n = 6). ***p < 0.001.

The drug toxicity of EAED is very low at the tissue level

Next, we analyzed the toxicity of EAED on mouse tracheal rings. After the 3.16 mg/mL EAED completely blocked the contraction induced by ACH, the tracheal rings were eluted and balanced for a while, again with ACH stimulation, and the contraction apparently occurs again (Figure 8A). Statistics show that the second ACH-induced shrinkage was approximately 81% of the first (Figure 8B). The above results showed that EAED had little effect on the activity of trachea rings when relaxing them, and could be used in vivo experiments.

Figure 8. The tracheal rings could still be stimulated to shrink after relaxation by EAED. (A) After 3.16 mg/mL EAED was added to inhibit the contraction by ACH, the tracheal rings were stimulated to shrink again by ACH. (B) The contraction rate after the first ACH stimulation and the second were compared. The statistics were obtained from the six tracheal rings.*** P < 0.001.

EAED reduces the respiratory resistance induced by ACH in the control group and the asthma group

To investigate whether EAED could potentially improve airway hyperresponsiveness in mice, lung function of groups of healthy or asthmatic mice was assessed by FOT at baseline and following doubling concentrations of aerosolized ACH (3.125 - 50 mg/mL) dissolved with vehicle or EAED. The four experimental groups studied were indistinguishable under baseline conditions by FOT. When the ACH concentration was increased to 25 - 50mg/mL, the atomized EAED dissolved ACH significantly reduced the respiratory resistance of the control group and the asthma group compared with the vehicle group (Figure 9). As expected, the group of asthmatic mice demonstrated the ACH-sensitive hyperresponsiveness compared to control group, as illustrated especially after adding 25 and/or 50 mg/mL aerosol ACH.

Figure 9. EAED reduced the respiratory resistance induced by ACH in the control group and the asthma group. At the baseline level (B), there was no significant difference in respiratory resistance between the four groups. When the ACH concentration was added to 25 - 50 mg/mL, the atomized ACH dissolved with EAED significantly reduced the respiratory resistance of the control group and the asthma group compared with the vehicle group. (*p < 0.05 Asthma+Vehicle vs Asthma+EAED, **p < 0.01 Asthma+Vehicle vs Control+Vehicle, #p < 0.05 Control+Vehicle vs Control+EAED; ANOVA).

Discussion

In this study, we found that EAED could reduce both high K^+ - and ACH-induced precontractions in mouse TRs and lung slices, via inhibiting L-type Ca^{2+} channels and additional TRPC3 and/or STIM/Orai channels, respectively. Elevated cytoplasmic Ca^{2+} concentration caused by high K^+ and ACH was suppressed by EAED. And in vivo study, we found EAED effectively reduced the elevated R_{rs} induced by ACH in the healthy mice and the asthmatic mice.

As we mentioned at the beginning, β_2 AR-agonists were often used as the first-line bronchodilators to relieve asthma²³, but their use has been found to cause many side effects and high recurrence rates. So, The purpose of this study was to find bronchodilators among natural plant. We first extracted a component (EAED) from dandelion. To investigate whether EAED has the diastolic effect on the airway smooth muscle of mice, we employed two stimuli, high K^+ and ACH, to elicit mouse TRs precontractions and observed the effects of EAED. Experimental results showed that EAED could markedly antagonize both high K^+ - and ACH-induced TR contractions in mice, and the maximum relaxant efficiency reached almost 100% (Figs. 1 and 2). These data demonstrated that EAED possesses a relaxant potency against contractions induced by ACH/high K^+ stimulation.

We further investigated the underlying mechanism of the EAED-mediated relaxant effects on high K^+ -induced contraction. The experiments were conducted under extracellular 0 Ca^{2+} /2 mM Ca^{2+} conditions. High K^+ could cause membrane depolarization, leading to the activation of VDLCCs²⁴. In our study, high K^+ -induced contractions were completely abolished in a Ca^{2+} -free medium (Fig. 5A), suggesting that this type of contraction may be dependent on Ca^{2+} influx through VDLCCs. And EAED could completely block VDLCCs currents (Fig. 7A). These data indicated that EAED relaxed high K^+ -induced contraction by blocking VDLCC-mediated Ca^{2+} influx (Fig. 5A and B).

We then explored the pathways involved in EAED-mediated relaxation of ACH-induced contractions. We found nifedipine partially inhibited ACH-induced sustained contractions under 2 mM Ca^{2+} conditions but had no effect on ACH-induced transient contractions under 0 Ca^{2+} conditions (Fig. 4A and 5C). These results suggested that VDLCCs were responsible for extracellular Ca^{2+} influx-triggered long-lasting contractions, but not intracellular Ca^{2+} releasing-induced transient contractions. However, EAED almost eliminated both of the two types of contractions (Fig. 5D), which suggested that the relaxant effects of EAED depended on blocking both the extracellular Ca^{2+} influx-mediated by VDLCCs and intracellular Ca^{2+} releasing.

Moreover, TRPC3 and/or STIM/Orai channels, as NSCCs channels, also play roles in ACH-induced contractions via mediating Ca^{2+} influx²⁵. Our results indicate that Pyr3 can also cause partial inhibition in the presence of nifedipine, which proves that TRPC3 and/or STIM/Orai channels are also involved in the contraction process. And NSCCs current was effectively blocked by EAED (Fig. 7B). However, in addition to L-type Ca^{2+} channels and NSCCs channels, there are still other channels that mediate ACH-

induced contractions being further inhibited by EAED (Fig. 4C). These unknown mechanisms required further investigation.

The above experimental results were all studied from the main trachea of mice. And then our experiments conducted in lung slices suggested that EAED could also inhibit contraction of bronchial smooth muscle (Fig. 3), indicating that EAED were able to block whole ASM contraction. In terms of Ca^{2+} dynamics in the ASMCs, we further showed that EAED decreased high K^+ and ACH-mediated increasing of intracellular Ca^{2+} (Fig. 6). In addition, in vivo study, we found that EAED could relieve respiratory resistance of healthy and asthmatic mice (Fig. 9).

Conclusion

In summary, the present study demonstrated that EAED can inhibit agonist-induced sustained contractions of ASM by inhibiting several types of ion channels, decrease agonist-induced increasing cytosolic free Ca^{2+} concentration in ASMCs and relieve respiratory resistance of healthy and asthmatic mice. Meanwhile, unknown pathways might also be involved in EAED-mediated relaxation in addition to VDCCs and NSCCs channels. These results suggest that EAED could be a new inhibitor of asthma attacks.

Abbreviations

EAED: Ethyl acetate extract from Dandelion

ASM: Airway smooth muscle

ASMCs: Airway smooth muscle cells

FOT: Forced oscillation technique

TMHM: *Taraxacum mongolicum* Hand.-Mazz.

LPS: Lipopolysaccharide

ACH: Acetylcholine chloride

Pyr3: pyrazole3

SPF: Specific pathogen free

Rrs: Respiratory system resistance

Declarations

Ethics approval and consent to participate

All methods applied in this study are in accordance with protocols approved by the South-Central University for Nationalities. All mice animal experiments were approved and performed under the supervision of the Institutional Animal Care and Use Committee of the South-Central University for Nationalities.

Consent for publication

Not applicable.

Competing Interests

The authors declare no competing Interests.

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Availability of data and materials

The data and materials supporting the conclusions are included within the article and its supplementary information files.

Authors' contributions

PZ and JL contributed equally to this study. PZ contributed to study design and guidance. JL conducted the experiments and wrote the manuscript. JWH and ZWY participated in scientific assistance. JPD provided Vibratome. DT and QM participated in data interpretation. JHS, QHL, PZ, and XZY supervised this study and edited the manuscript.

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Figures

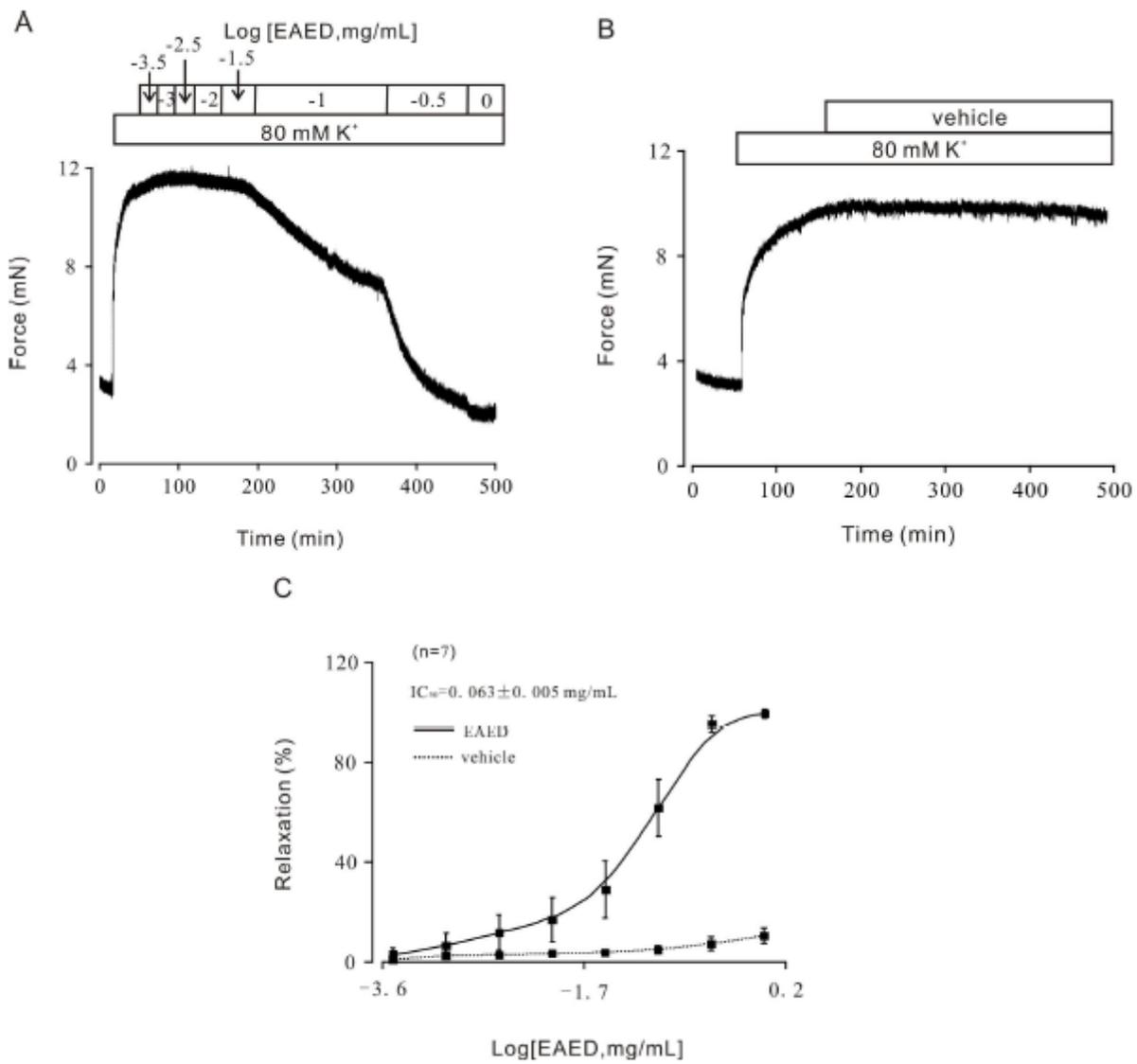


Figure 1

EAED inhibited high K⁺-induced tracheal ring contraction.

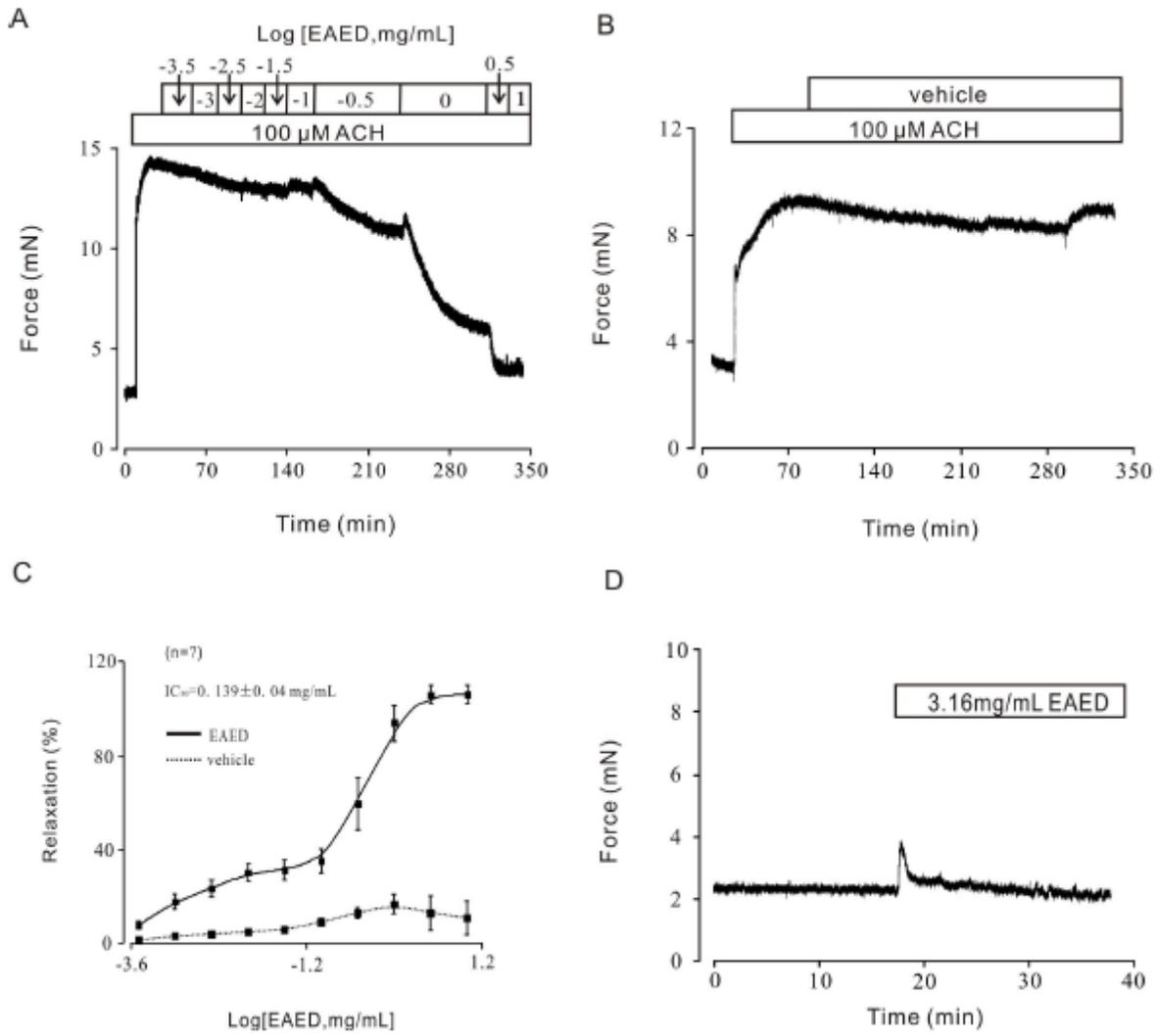


Figure 2

Contraction induced by ACH was inhibited by cumulative addition of EAED.

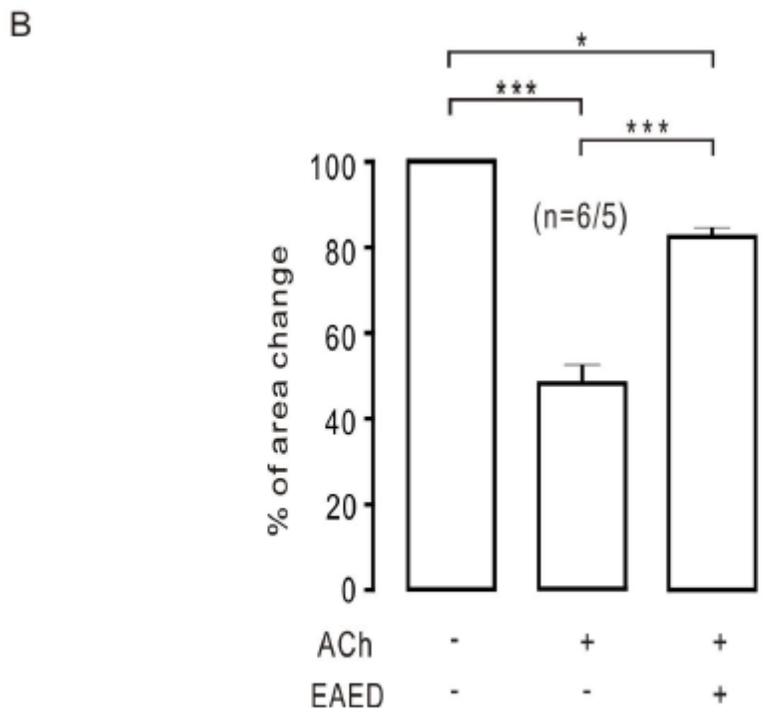
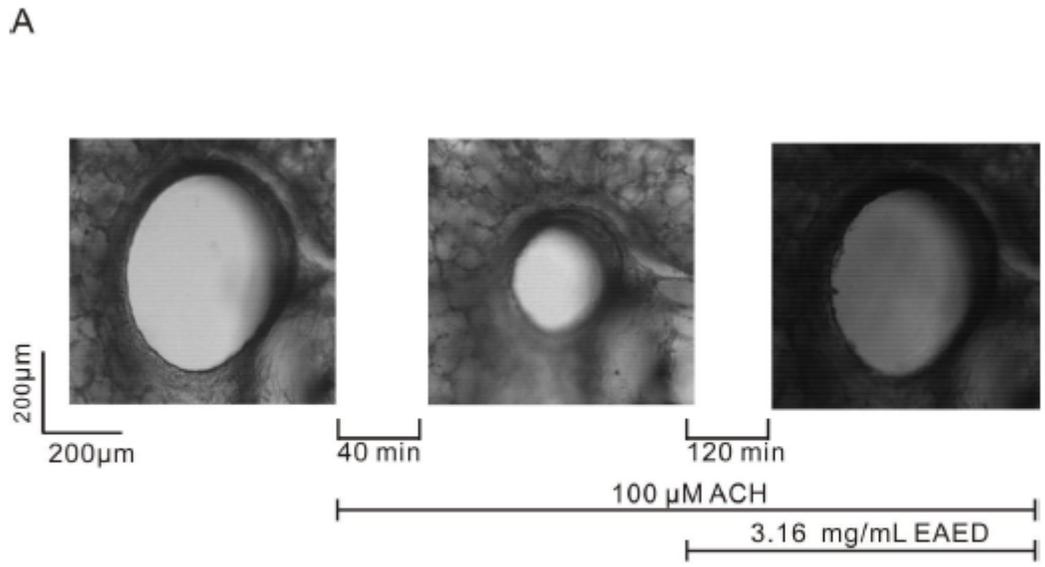


Figure 3

EAED inhibits contraction in lung slices.

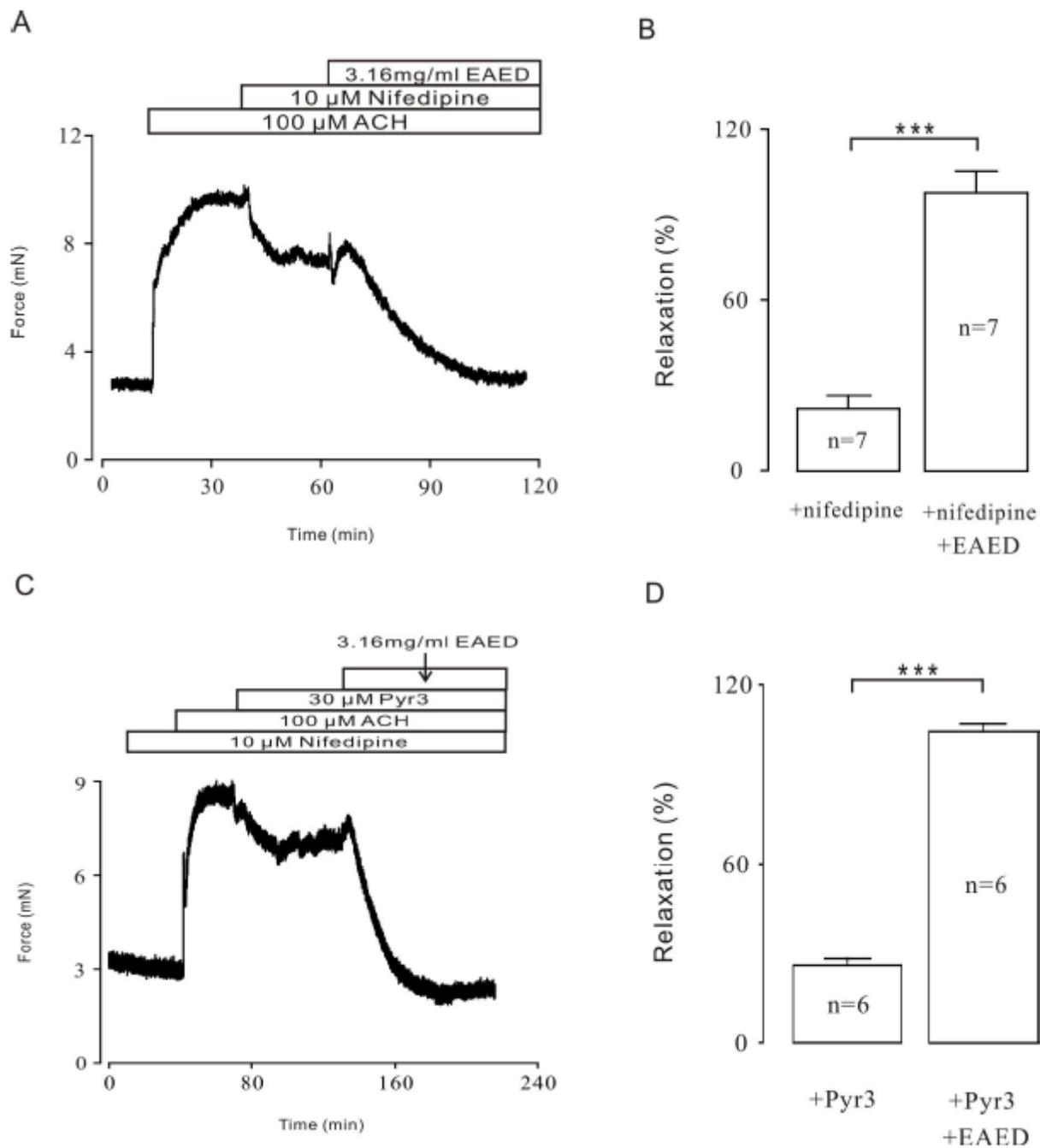


Figure 4

Nifedipine, Pyr3 partially inhibits ACh-induced contraction, respectively.

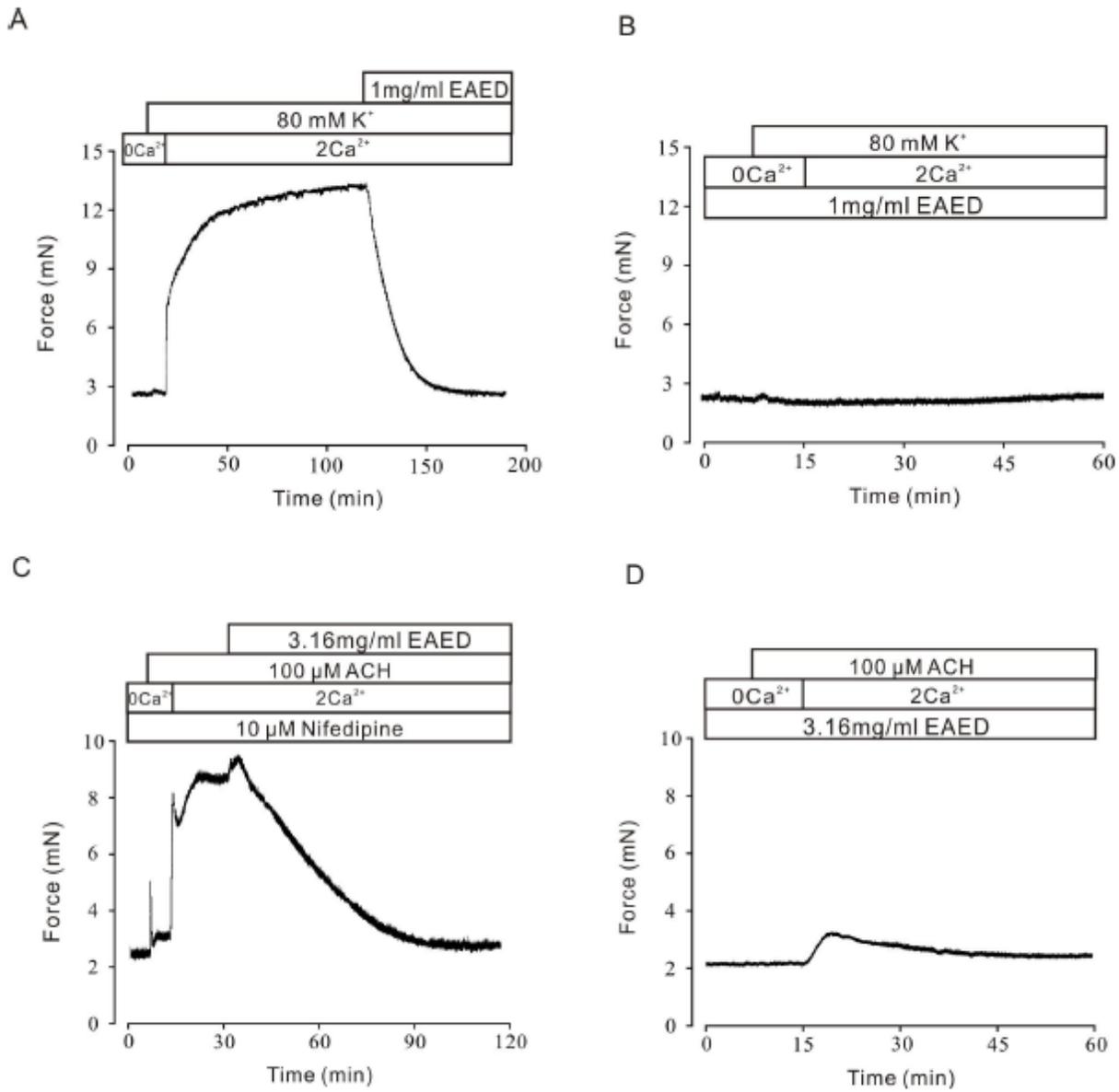


Figure 5

EAED blocks high K⁺-evoked Ca²⁺ influx and ACH-elicited Ca²⁺ influx and Ca²⁺ release.

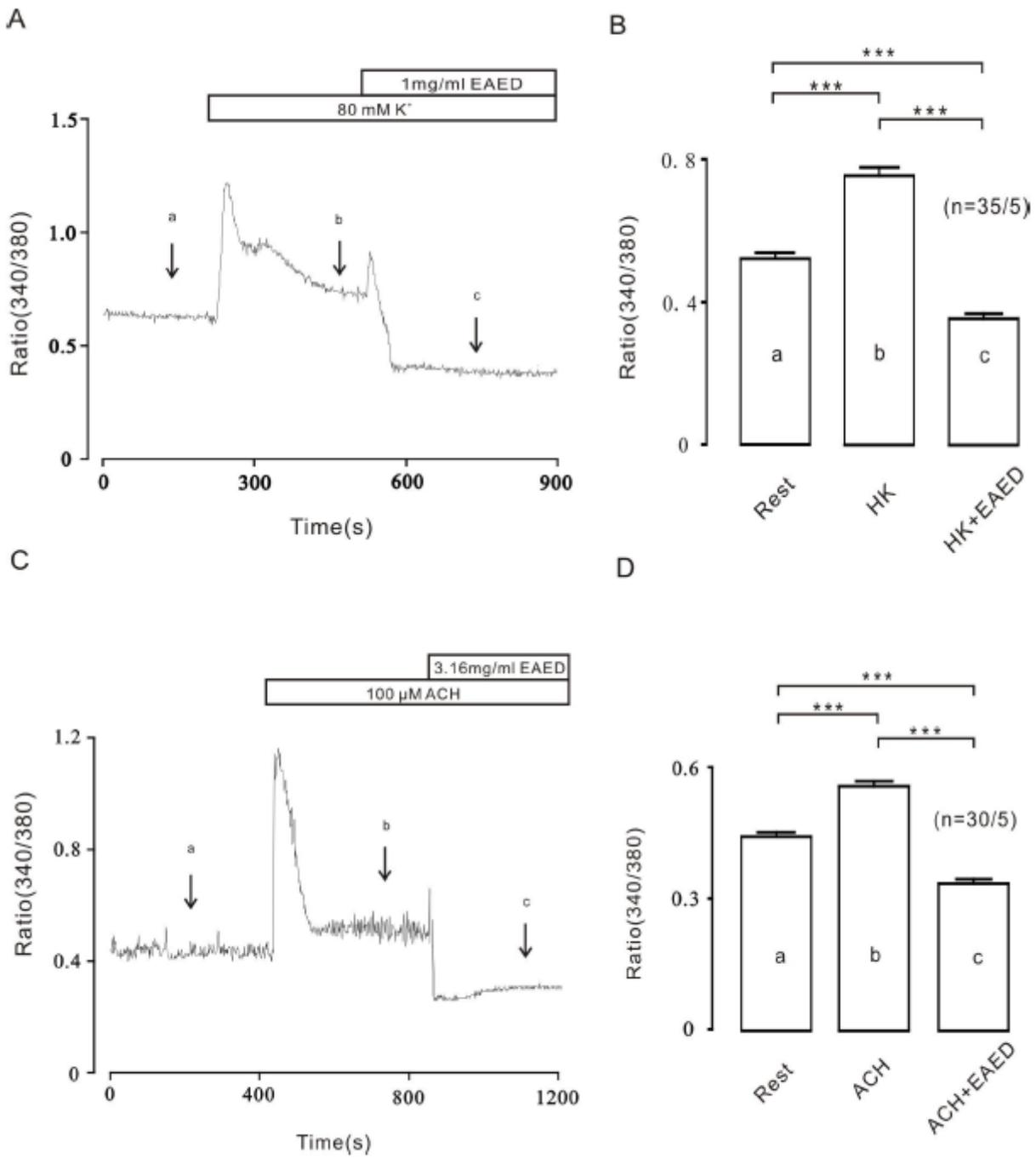


Figure 6

EAED inhibits high K⁺ and ACH-induced Ca²⁺ increases in single tracheal smooth muscle cells.

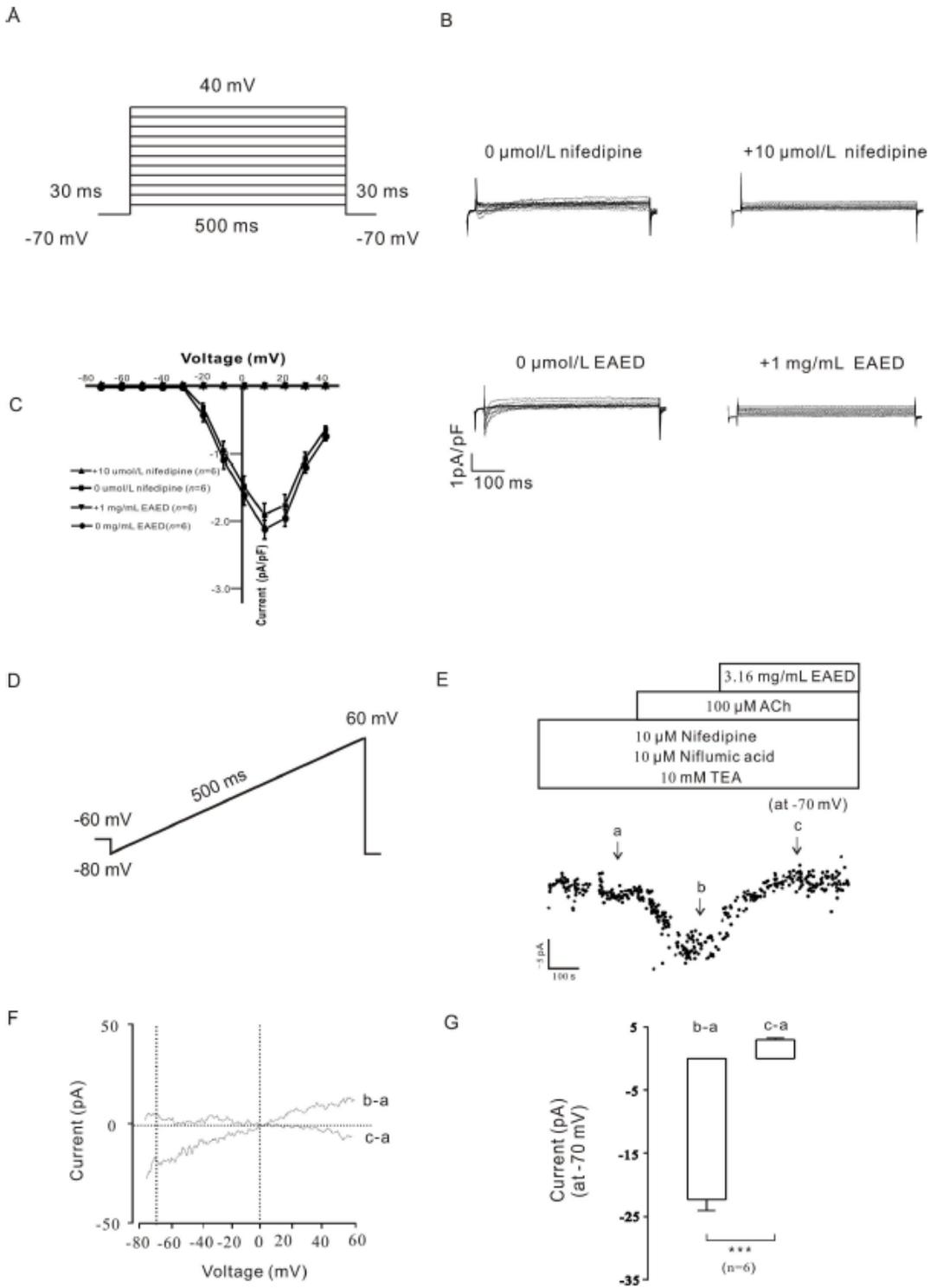


Figure 7

EAED blocks VDLCCs and NSCCs currents.

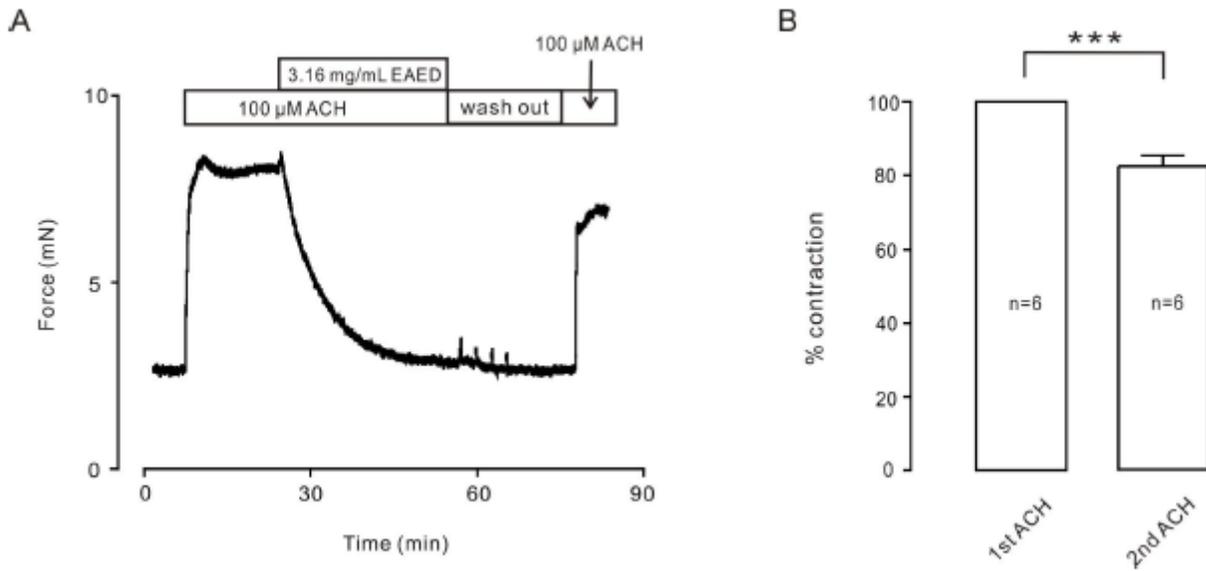


Figure 8

The tracheal rings could still be stimulated to shrink after relaxation by EAED.

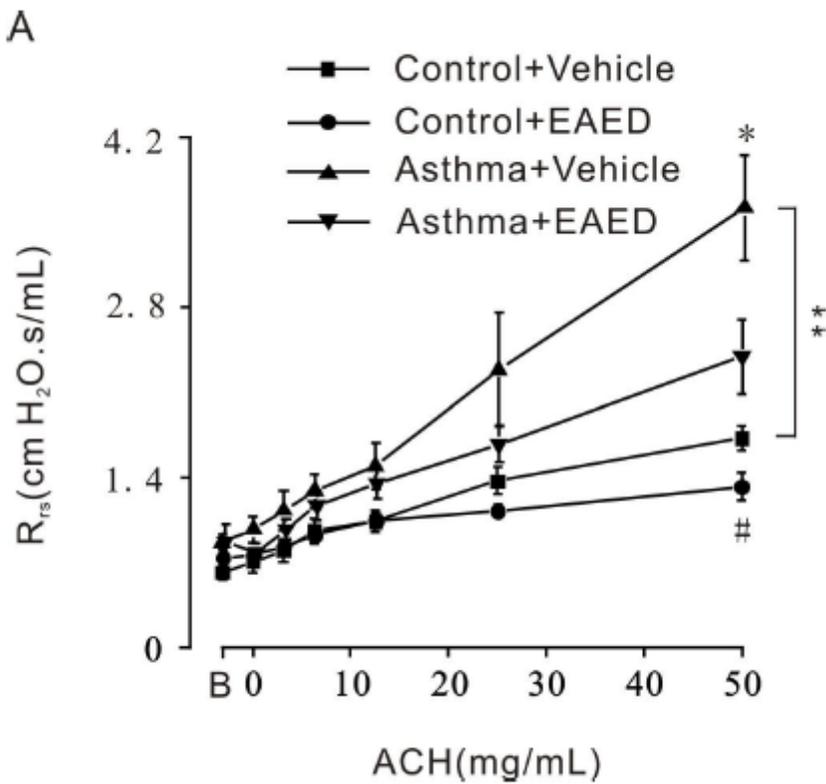


Figure 9

EAED reduced the respiratory resistance induced by ACH in the control group and the asthma group.