

Title: Effects of neostigmine on bronchoconstriction with continuous electrical stimulation in rats

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Keywords: neostigmine, continuous electrical stimulation, rat trachea,
asthma

Word count: 2961

Number of tables: 0

Number of figures: 7

Abstract

Purpose. When neostigmine is used to reverse muscle relaxants in patients with asthma without signs of airway inflammation, asthma attack is encountered occasionally. It is likely that abnormally-increased electrical impulses traveling from brain through cholinergic nerves to airway smooth muscles may be one of the pathogeneses of asthma attack. We applied continuous electrical field stimulation (c-EFS) or continuous electrical stimulation (c-ES) with low frequency to vagal nerve of the rat *in vitro* and *in vivo* to determine the role of cholinergic nerve activation in inducing airway constriction.

Methods. Fifty-seven male Wistar rats were used. In *in vitro* study we examined whether tetrodotoxin (TTX), a Na⁺ channel blocker, 4-DAMP, a muscarinic M₃ receptor antagonist, or neostigmine could affect c-EFS-induced contraction of the tracheal ring. In *in vivo* study, we examined whether c-ES of vagal nerve could increase maximum airway pressure (Pmax), and neostigmine could potentiate c-ES-induced Pmax.

Results. TTX and 4-DAMP completely inhibited c-EFS-induced contraction, while neostigmine potentiated c-EFS-induced contraction dose-dependently. Pmax was not increased by neostigmine.

Pmax was not increased by 2-Hz c-ES, but increased by the addition of

neostigmine. Pmax was increased by 5-Hz c-ES, and further increased by the addition of neostigmine.

Conclusion. The contractile response of the tracheal ring to c-EFS is potentiated by neostigmine. Pmax is increased by c-ES of vagal nerve, and is potentiated by neostigmine. These data suggest that the increased activity of the cholinergic nerve would play a role in asthma attack.

Introduction

When neostigmine is used to reverse muscle relaxants in patients with asthma without signs of airway inflammation, asthma attack is encountered occasionally. Hoang BX et al. [1] reported that although airway inflammation is now widely accepted as the pathogenesis of asthma, many patients show no signs of inflammation, yet they still have severe airflow limitation and asthma symptoms. Jain S and Jain KC [2] reported that the efficacy of phenytoin, antiepileptic drug, for the relief of chronic asthma is evaluated in an open trial in asthmatics, and they indicated that phenytoin is a useful anti-asthmatic agent used either alone, or as adjuvant therapy. Sayar B and Polvan O [3] suggested that certain bronchial asthma might well be considered as epileptic equivalents. Thus, it is likely that abnormally-increased electrical impulses traveling from brain through cholinergic nerves to airway smooth muscles may be one of the pathogeneses of asthma attack during the reversal of muscle relaxants by neostigmine. We applied continuous electrical field stimulation (c-EFS) to rat tracheal rings *in vitro* and also applied continuous electrical stimulation (c-ES) with low frequency to the vagal nerve of the rat *in vivo*, and examined the effects of c-EFS or c-ES on the contractile response and the maximum airway pressure (Pmax) in the absence or presence of neostigmine to

determine the role of cholinergic nerve activation in inducing airway constriction.

Materials and methods

This study was conducted following guidelines approved by our Institutional Animal Care Committee.

In vitro study

Twenty-nine male Wistar rats (Charles River, Yokohama, Japan) weighing 250-350 g were used for the experiments. The rats were exsanguinated under anesthesia with pentobarbital (50 mg/kg intraperitoneal), and the trachea was rapidly isolated.

Each trachea was cut into 3-mm-wide ring segments with a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, UK). We used only the distal three or two rings of the trachea. In each experiment, eight rings from three rats were used in eight organ chambers. The tracheal ring was suspended between two stainless steel hooks and placed in a 5-mL water-jacketed organ chamber (Kishimotoika, Kyoto, Japan) containing Krebs-Henseleit (K-H) solution (mM composition: NaCl 118, KCl 4.7, CaCl₂ 1.3, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, glucose 11, Na₂-EDTA 0.05). The solution was continuously aerated with O₂ 95%/CO₂ 5% at 37°C. Isometric tensions were measured using an isometric transducer (Kishimotoika, Kyoto, Japan) and changes in isometric force were recorded using a PowerLab system (ADInstruments Pty Ltd, Bella Vista, Australia).

The resting tension was periodically adjusted to 1.0 g during the equilibration period. The rings were washed every 15 min and re-equilibrated to baseline tension for 60 min (Time 0).

Electrical stimuli were generated by an electrical stimulator (SEN-7203; Nihon Kohden, Tokyo, Japan) and applied between two platinum electrodes. c-EFS was defined as an electrical stimulation for more than 60 sec, while an ordinary EFS was as an electrical stimulation for 10 sec [4, 5].

First, we examined whether c-EFS causes frequency-dependent airway smooth muscle contraction. Rectangular pulses (pulse duration 2 ms and 25 V) of 2 to 25 Hz were delivered continuously to the fields from the electrical stimulator.

Second, we examined whether c-EFS-induced contraction is sustained for several hours. Pulses (2 ms, 25 V, 5 Hz, which is the potency to induce under 50% of the level of maximal contraction) were delivered continuously for 180 min.

Third, we examined whether the magnitude of contraction induced by c-EFS is comparable to that by ordinary 10-sec EFS. Pulses (2 ms, 25 V and 5 Hz) were delivered for 10 sec and 5 min later, the same pulses were delivered continuously for 10 min.

Fourth, we examined whether tetrodotoxin (TTX; a Na⁺ channel blocker),

4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; a muscarinic M_3 receptor antagonist), or neostigmine affects c-EFS-induced contraction. Pulses (2 ms, 25 V and 5 Hz) were delivered continuously, and 5 min later, TTX (0.01, 0.03, 0.1, 0.3 and 1 μM), 4-DAMP (0.001, 0.003, 0.01, 0.03 and 0.1 μM) or neostigmine (0.1, 1 and 10 μM) were added in final concentrations.

In vivo study

Twenty-eight male Wistar rats weighing 300-380 g were used for the experiments. The rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and catheter was inserted into the trachea, and then artificially ventilated with a small animal ventilator (Model SAR-830, CEW, Inc., Ardmore PA, USA) with O_2 100% at rate of 40 br/min. We set the ventilator to make the initial airway pressure 10 mmHg with adjusting inspiratory flow. Catheters in the left carotid artery and right jugular vein were inserted to assess blood pressure, and for delivery of drugs and supplementary fluid. Lactated Ringer solution containing pentobarbital (0.0625 mg/ml) and vecuronium (0.05 mg/ml) was continuously infused at a rate of 10 ml/kg/h by an infusion pump (Coopdech CSP-100, Daiken-ika, Osaka, Japan). Electrical stimuli were generated by the electrical stimulator and applied to the right vagal nerve in cervical region between two platinum electrodes.

c-ES was defined as an electrical stimulation for more than 60 sec. Airway pressure was measured by a pressure transducer connected to PowerLab.

When P_{max} was adjusted to 10 mmHg and stabilized for 5 min, this pressure was determined as the baseline pressure (Time 0).

First, we examined whether 2- or 5-Hz c-ES causes the increase in P_{max}, and neostigmine potentiates the c-ES-induced increase in P_{max}.

Rectangular pulses (2 ms, 25 V) of 2 or 5 Hz were delivered continuously to the vagal nerve from the electrical stimulator, and 1 min later neostigmine (0.05 mg/kg) was injected to the jugular vein.

Second, we examined whether the pre-injection of neostigmine affects c-ES-induced increase in P_{max}. Neostigmine (0.05 mg/kg) was injected, and 2 min later pulses (2 or 5 Hz) were delivered continuously.

Data analyses

Data were expressed as mean \pm SD. Concentration- or frequency- effect curves were fitted by nonlinear regression (GraphPad Prism; GraphPad, San Diego, CA, USA). The results were analyzed by ANOVA followed by Tukey's Multiple Comparison Test. A value of $P < 0.05$ was considered statistically significant.

Results

The 2- to 25-Hz c-EFS induced the contraction of the tracheal ring frequency-dependently (Fig.1).

Five-Hz, which induces nearly 50% maximal contraction was delivered continuously for 180 min. The 5-Hz c-EFS-induced contraction increased in the strength, and was sustained at nearly same levels for 60 min, and then gradually decreased. After completion of stimulations, contraction quickly decreased to the baseline (Fig. 2).

5-Hz EFS was delivered for 10 sec and 5 min later, 5-Hz c-EFS was delivered for 10 min. c-EFS-induced contraction was 4 folds greater than that induced by 10-sec EFS (Fig. 3).

TTX and 4-DAMP completely inhibited c-EFS-induced contraction (Fig. 4, Fig.5), while neostigmine potentiated c-EFS-induced contraction dose-dependently (Fig. 6), suggesting that the contractile response to c-EFS is mediated by acetylcholine (ACh) released from the cholinergic nerve terminals.

In Fig.7 Pmax was not increased by 2 Hz c-ES, but increased by addition of neostigmine. Pmax was increased by 5 Hz c-ES, and also increased by addition of neostigmine. Pmax was not increased by neostigmine but was increased by addition of 2 and 5 Hz c-ES.

Discussion

In the present *in vitro* study with rat tracheal rings, we have observed that tracheal smooth muscle tension is more responsive to c-EFS than to ordinary EFS for 10 sec, and that c-EFS-induced contraction is inhibited by TTX and 4-DAMP while is potentiated by neostigmine.

Since spontaneous depolarization is occurred at a frequency of approximately one per second [6], electrical stimulations at low-frequency used in the present study seem to be physiological.

Parasympathetic postganglionic neurons are considered to be close to the targeted end-organ, tracheal rings used in the present study would contain parasympathetic postganglionic neurons. TTX is used as the denervating drug in the isolated tracheal experiments. The effects of c-EFS and the role of parasympathetic postganglionic neurons were examined with TTX. Toda et al. reported in the isolated trachea that since ordinary EFS-induced response was abolished by TTX 0.1 μ M, cholinergic nerves would mediate the contractile response to ordinary EFS [7]. In the present study, c-EFS-induced contraction was blocked completely by TTX, 0.1 μ M, suggesting that c-EFS-induced contraction would be mediated by nervous system but not by direct stimulation of airway smooth muscle.

Muscarinic ACh receptors in the airway are divided into M_2 and M_3

receptors [8]. The muscarinic M_3 receptors exist on airway smooth muscle cell membrane. Stimulation of M_3 receptors induces airway smooth muscle contraction. In the present study, c-EFS-induced contraction was nearly inhibited by 4-DAMP, 30 nM. Ten Berge et al.[9] tested the effects of 4-DAMP on the twitch response of ordinary electrical field-stimulated guinea pig tracheal ring preparations, and found that twitch contraction was nearly inhibited by 4-DAMP at a dose of 10 nM. Their result is consistent with our data. Thus, in the present study the contractile response to c-EFS would be mediated via muscarinic M_3 receptors.

When the contraction strengths were compared at 5Hz, c-EFS-induced contraction was 4 times greater than contraction induced by 10-sec EFS, and c-EFS-induced contraction was maintained for 3 hr. Kirkpatrick and Rooney [10] reported that when tracheal muscle strips were stimulated electrically at supramaximal pulses of 50 Hz, contractile responses continued for 1 hr, and the contraction was well maintained during the period of ES. Our present results suggest that when EFS is added continuously to cholinergic nerves, contraction would be enhanced even in low frequency and be lasting, and yet significant depletion of ACh stores would not take place during c-EFS.

In the present *in vivo* study, Pmax was not increased by neostigmine. In our previous *in vitro* study even maximal concentration of edrophonium, an

anticholinesterase drug did not cause a contractile response [11]. The lack of effect of edrophonium suggests that there is no spontaneous release of ACh from nerve terminals in the rat trachea. Thus, it is likely that there is no spontaneous release of ACh in the airway of rat in the present *in vivo* study.

Pmax was not increased by 2-Hz c-ES of the vagal nerve, but increased by addition of neostigmine. The mechanism involved in the enhancing effect of neostigmine on the c-ES-induced Pmax may be advanced as follows. When c-ES is applied to cholinergic nerves, depolarization of the terminal and subsequent Ca^{2+} influx are induced. The Ca^{2+} influx facilitates massive ACh release from the synaptic vesicles. Released ACh binds muscarinic M_3 receptors at airway smooth muscle, resulting in contraction, and then ACh is rapidly hydrolyzed by choline esterase to choline and acetic acid. It is likely that 2-Hz c-ES alone would not release ACh enough to induce bronchoconstriction. However, in the presence of neostigmine, ACh accumulates at the endplate of airway smooth muscle, resulting in the increase in Pmax.

The dose of neostigmine used in the present *in vivo* study is clinically relevant. Hazizaj and Hatija [12] reported the case of bronchospasm caused by neostigmine. Gouge SF et al. [13] gave pyridostigmine, an anti-cholinesterase drug to asthmatics and found no changes in forced vital

capacity in any of them, but observed exacerbation of asthma symptoms.

Although the frequency of impulses required for bronchoconstriction is not clear, our results suggest that 2 impulses or more at a second would be enough to excite the cholinergic nerve to cause airway constriction.

Asthma attack is encountered occasionally when neostigmine is used in patients with asthma. Since sugammadex can be used in clinical setting now, neostigmine for the reversal of muscle relaxants should not be used as before in patients with asthma.

In conclusion, the contractile response of the tracheal ring to c-EFS is potentiated by neostigmine. Pmax is increased by c-ES of vagal nerve, and is potentiated by neostigmine. These data suggest that the increased activity of the cholinergic nerve would play a role in asthma attack.

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Legends

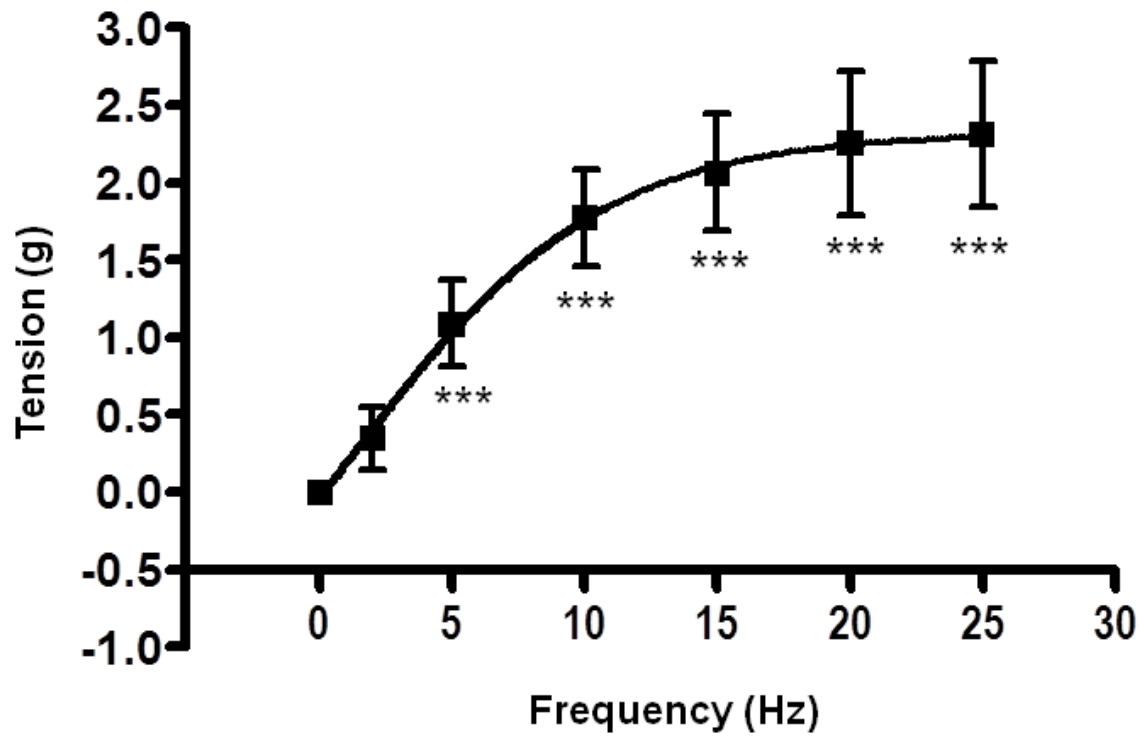


Fig. 1.

The effects of frequency on c-EFS-induced tension of rat tracheal rings (mean \pm SD, n = 6). c-EFS: continuous electrical field stimulation.

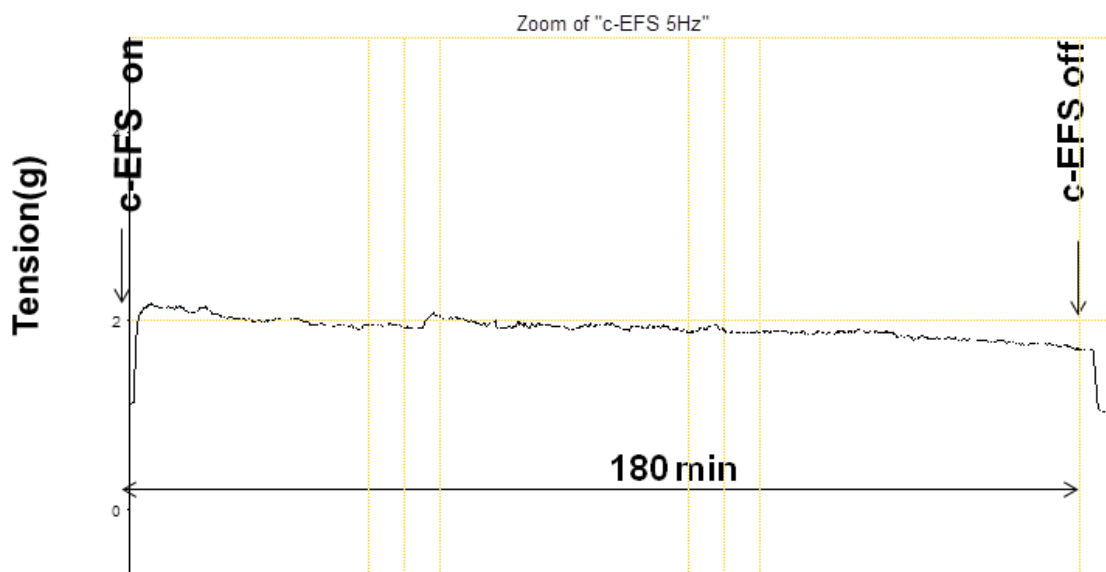


Fig. 2.

A recording of time course of c-EFS-induced contraction of the rat tracheal ring. Tension was measured by additions of 5-Hz c-EFS for 180 min. c-EFS: continuous electrical field stimulation.

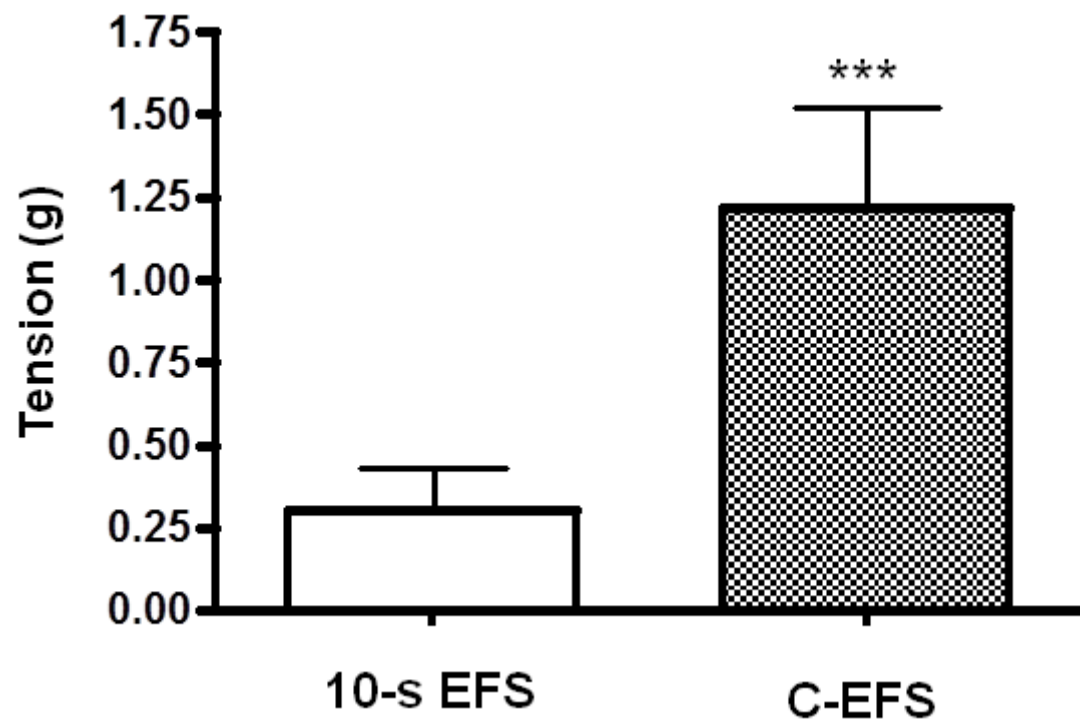


Fig. 3.

The effects of 5-Hz 10-sec EFS- and 5-Hz c-EFS-induced contraction of the rat tracheal rings (mean \pm SD, n = 6). *** P < 0.001 vs 5-Hz 10-sec EFS. c-EFS: continuous electrical field stimulation.

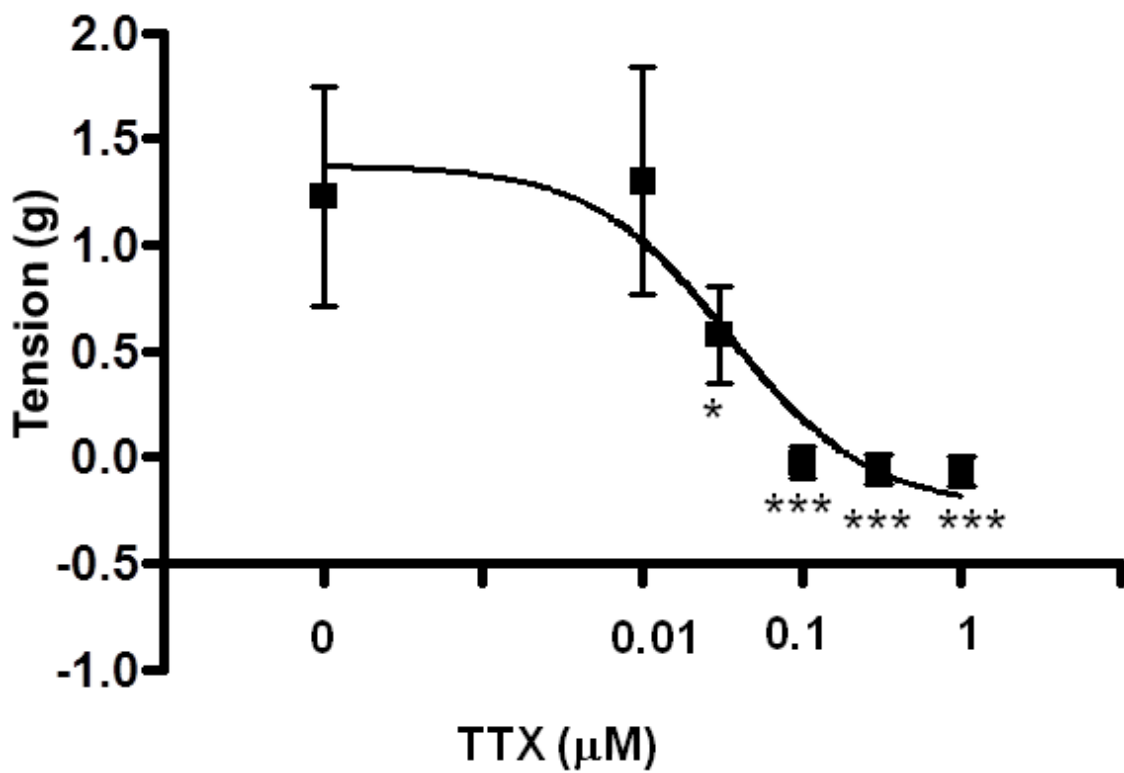


Fig. 4.

The effects of tetrodotoxin (TTX) on 5-Hz c-EFS-induced contraction of the rat tracheal rings (mean \pm SD, n = 6). * P < 0.05; *** P < 0.001 vs TTX

0. c-EFS: continuous electrical field stimulation.

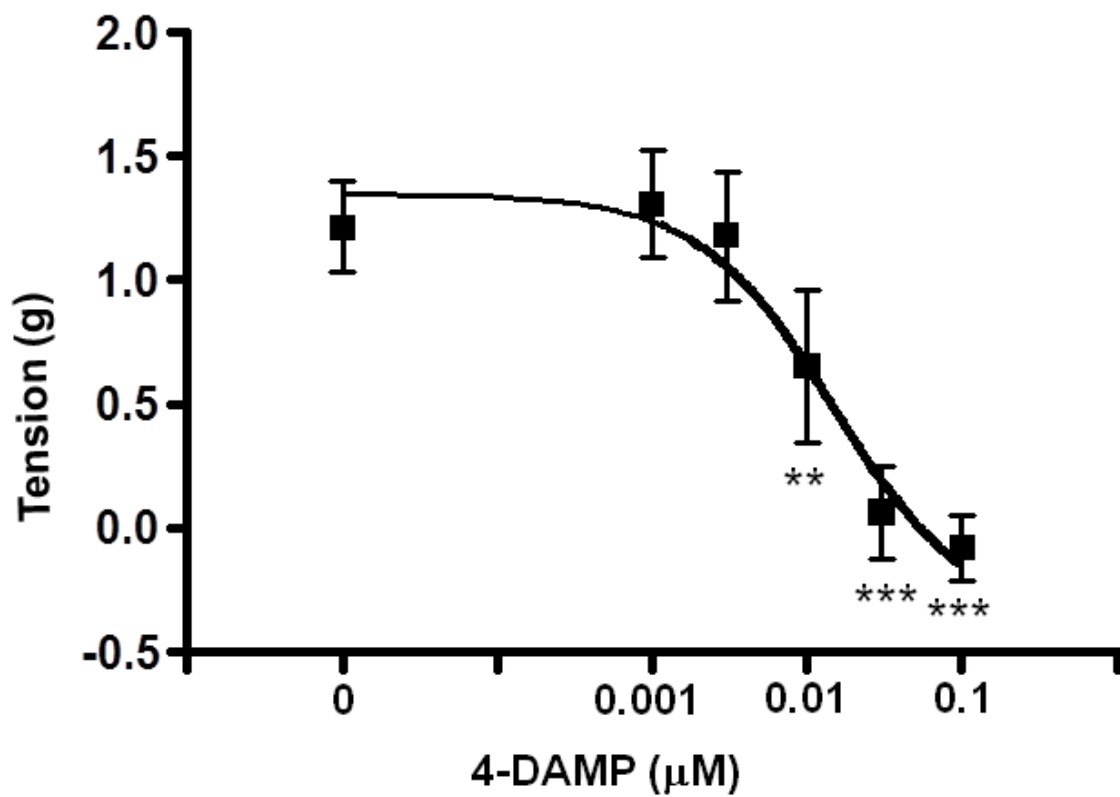


Fig. 5.

The effects of 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) on 5-Hz c-EFS-induced contraction of rat tracheal rings (mean \pm SD, n = 7). ** P < 0.01; *** P < 0.001 vs 4-DAMP 0. c-EFS: continuous electrical field stimulation.

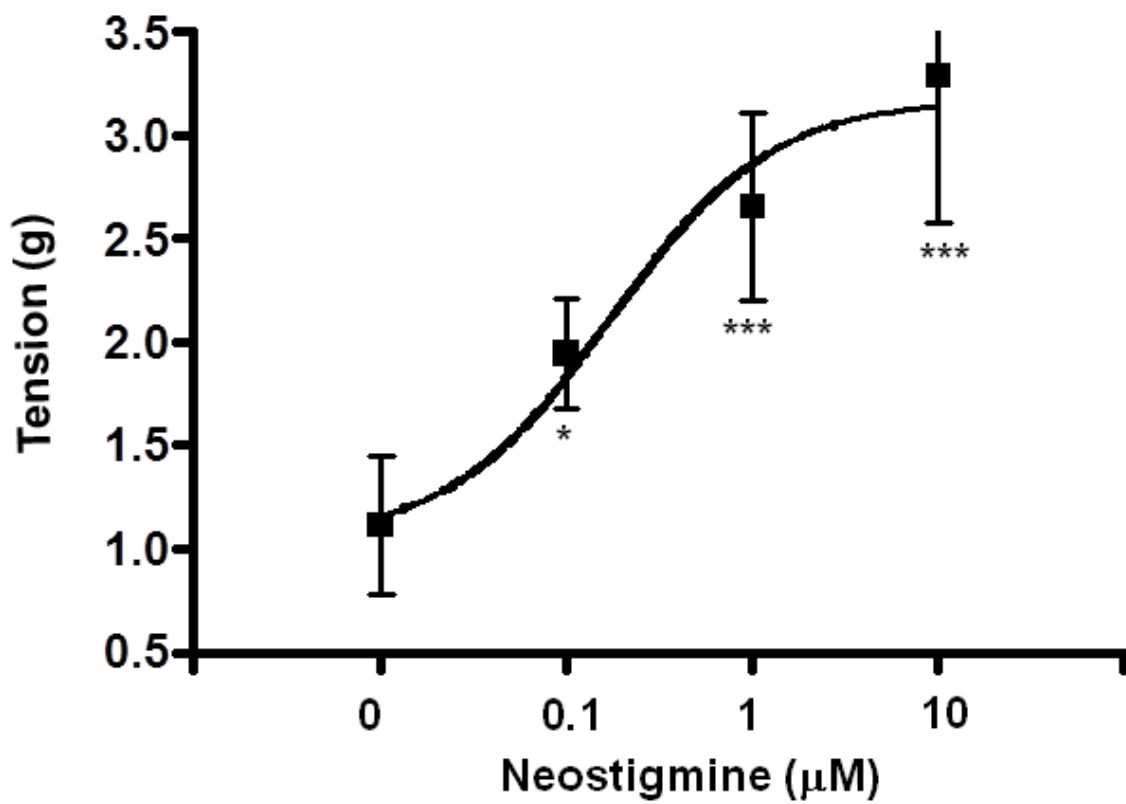


Fig. 6.

The effects of neostigmine on 5-Hz c-EFS-induced contraction of rat tracheal rings (mean \pm SD, n = 6). * P < 0.05; *** P < 0.001 vs neostigmine 0. c-EFS: continuous electrical field stimulation.

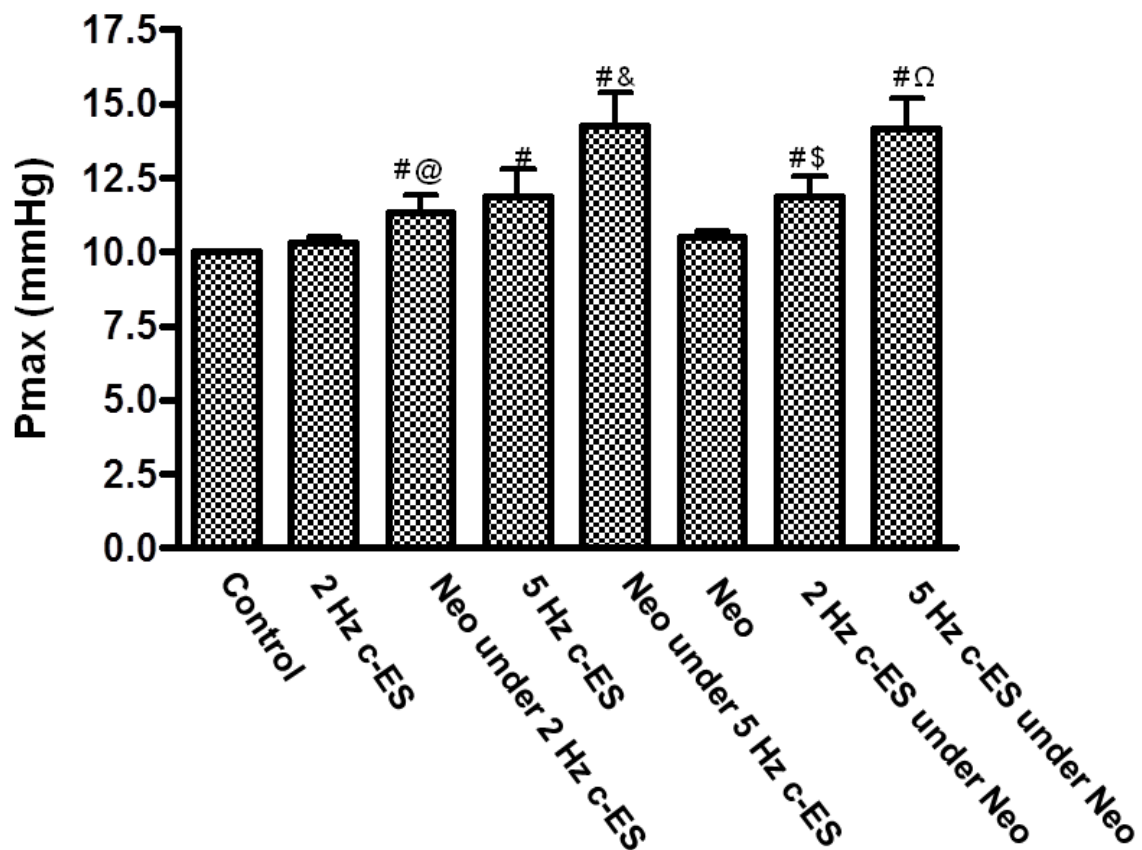


Fig. 7

1) The effects of 2-Hz c-ES and neostigmine under 2-Hz c-ES on maximum airway pressure (Pmax) in rats (mean \pm SD, n = 6). Pmax was not increased by 2-Hz c-ES, but increased by addition of neostigmine .

2) The effects of 5-Hz c-ES and neostigmine under 5-Hz c-ES on Pmax in rats (mean \pm SD, n = 6). Pmax was increased by 5-Hz c-ES, and also increased by addition of neostigmine.

3) The effects of neostigmine and 2-Hz c-ES under addition of neostigmine on Pmax in rats (mean \pm SD, n = 8). Pmax was not increased by neostigmine but increased by addition of 2-Hz c-ES.

4) The effects of neostigmine and 5-Hz c-ES under addition of neostigmine on Pmax in rats (mean \pm SD, n = 7). Pmax was not increased by neostigmine but was increased by addition of 5-Hz c-ES.

P < 0.001 vs Control. @ P < 0.001 vs 2-Hz c-ES. & P < 0.001 vs 5-Hz c-ES. \$ P < 0.01 vs Neo. Ω P < 0.001 vs Neo. c-ES: continuous electrical stimulation. Neo: neostigmine.