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ORIGINAL ARTICLE

Effects of neostigmine on bronchoconstriction with continuous electrical stimulation in rats

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Abstract
Purpose When neostigmine is used to reverse muscle relaxants in patients with asthma without signs of airway inflammation, asthma attack is occasionally encountered. It is likely that abnormally increased electrical impulses traveling from the 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) of low frequency to the 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 an in vitro study we examined whether tetrodotoxin (TTX), an Na^+-channel blocker, 4-DAMP, a muscarinic M_3 receptor antagonist, or neostigmine could affect c-EFS-induced contraction of the tracheal ring. In an in vivo study, we examined whether c-ES of the vagal nerve could increase maximum airway pressure (P_{max}) and whether neostigmine could potentiate c-ES-induced P_{max}.
Results TTX and 4-DAMP completely inhibited c-EFS-induced contraction whereas neostigmine potentiated c-EFS-induced contraction dose-dependently. P_{max} was not increased by neostigmine. P_{max} was not increased by 2-Hz c-ES, but was increased by the addition of neostigmine. P_{max} 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. P_{max} is increased by c-ES of the vagal nerve, and is potentiated by neostigmine. These data suggest that increased activity of the cholinergic nerve could be involved in asthma attack.

Keywords Neostigmine · Continuous electrical stimulation · Rat trachea · Asthma

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Introduction

When neostigmine is used to reverse muscle relaxants in patients with asthma without signs of airway inflammation, asthma attack is occasionally encountered. Hoang 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 still have severe airflow limitation and asthma symptoms. Jain and Jain [2] reported evaluation of the efficacy of phenytoin, an antiepileptic drug, for relief of chronic asthma in an open trial in asthmatics, and indicated that phenytoin is a useful anti-asthmatic agent used either alone or as adjuvant therapy. Sayar and Polvan [3] suggested that some bronchial asthma might well be regarded as epileptic equivalents. Thus, it is likely that abnormally increased electrical impulses traveling from the 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 (P_{max}) 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 in accordance with 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, $CaCl_2$ 1.3, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, Na_2-EDTA 0.05). The solution was continuously aerated with O_2 95%/CO_2 5% at 37°C. Isometric tension was measured using an isometric transducer (Kishimotoika, Kyoto, Japan) and changes in isometric force were recorded using a PowerLab system (ADInstruments, 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 electrical stimulation for more than 60 s whereas ordinary EFS was electrical stimulation for 10 s [4, 5].

First, we examined whether c-EFS causes frequency-dependent airway smooth muscle contraction. Rectangular pulses (pulse duration 2 ms at 25 V) of 2–25 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 inducing <50% of maximum contraction) were delivered continuously for 180 min.

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

Fourth, we examined whether tetrodotoxin (TTX; an 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, or 1 μM), 4-DAMP (0.001, 0.003, 0.01, 0.03, or 0.1 μM), or neostigmine (0.1, 1, or 10 μM) were added at the final concentrations indicated.

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), catheters were inserted into the tracheas, and the animals were then artificially ventilated with a small animal ventilator (model SAR-830; CEW, Ardmore, PA, USA), with 100% O_2, at a rate of 40 br/min. We set the ventilator to make the initial airway pressure 10 mmHg by adjusting inspiratory flow. Catheters were inserted in the left carotid artery and right jugular vein to assess blood pressure and for delivery of drugs and supplementary fluid. Lactated Ringer's 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 use of 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 the cervical region between two platinum electrodes. c-ES was defined as electrical stimulation for more than 60 s. 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 an increase in P_{max}, and whether 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 pre-injection of neostigmine affects the 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 analysis

Data are expressed as mean ± SD. Concentration–effect 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 frequency-dependent contraction of the tracheal ring (Fig. 1).

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

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

TTX and 4-DAMP completely inhibited c-EFS-induced contraction (Figs. 4, 5) whereas 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.

Figure 7 shows that P_{max} was not increased by 2 Hz c-ES but was increased by addition of neostigmine. P_{max} was increased by 5 Hz c-ES, and also increased by addition

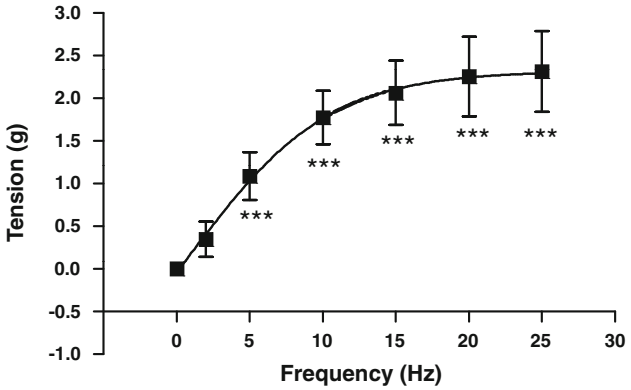


Fig. 1 Effect of frequency on c-EFS-induced tension of rat tracheal rings (mean ± SD, $n = 6$). *c-EFS* continuous electrical field stimulation

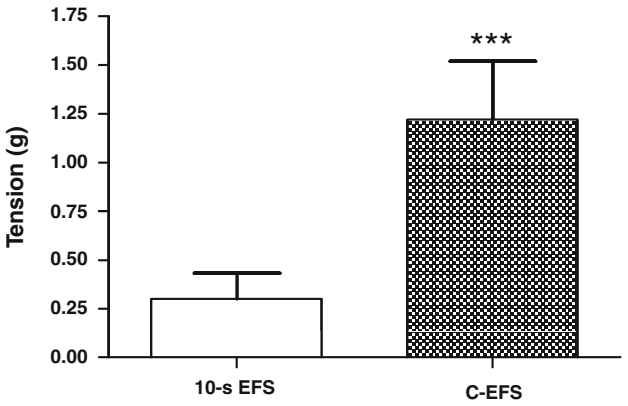


Fig. 3 Effects of 5-Hz 10-s EFS-induced and 5-Hz c-EFS-induced contraction of rat tracheal rings (mean ± SD, $n = 6$). ***$P < 0.001$ versus 5-Hz 10-s EFS. *c-EFS* continuous electrical field stimulation

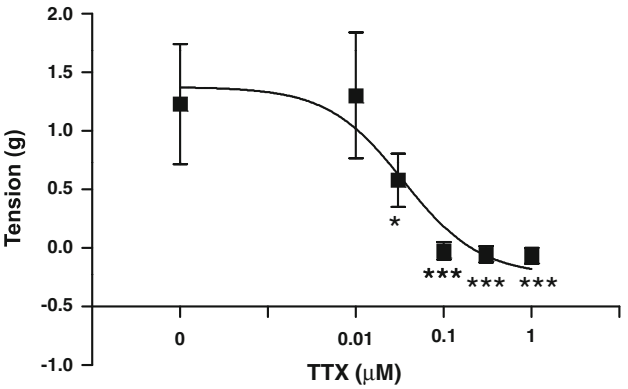
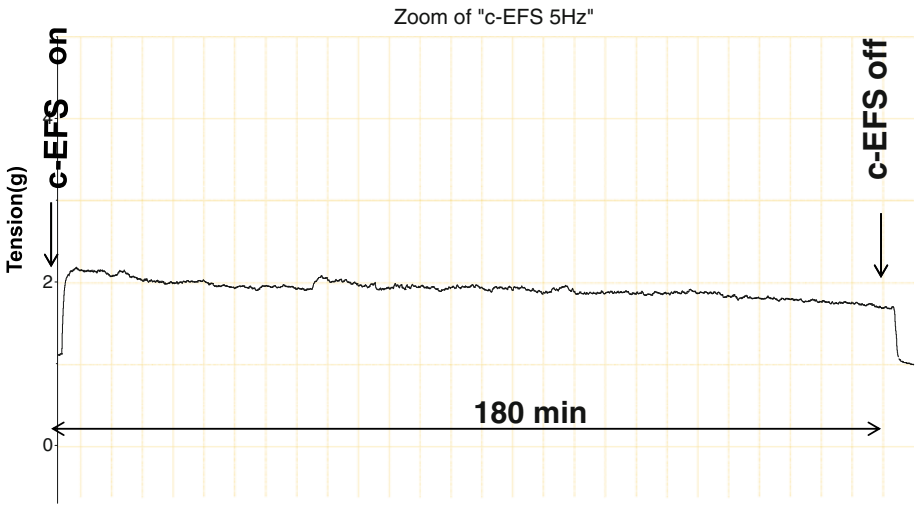


Fig. 4 Effect of tetrodotoxin (TTX) on 5-Hz c-EFS-induced contraction of rat tracheal rings (mean ± SD, $n = 6$). *$P < 0.05$; ***$P < 0.001$ versus TTX 0. *c-EFS* continuous electrical field stimulation

Fig. 2 Recording of the time course of c-EFS-induced contraction of a rat tracheal ring. Tension was measured during application of 5-Hz c-EFS for 180 min. *c-EFS* continuous electrical field stimulation



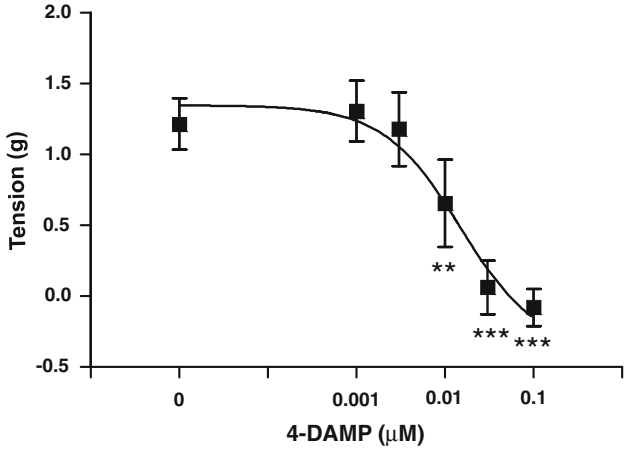


Fig. 5 Effect of 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) on 5-Hz c-EFS-induced contraction of rat tracheal rings (mean ± SD, $n = 7$). $^{**}P < 0.01$; $^{***}P < 0.001$ versus 4-DAMP 0. *c-EFS* continuous electrical field stimulation

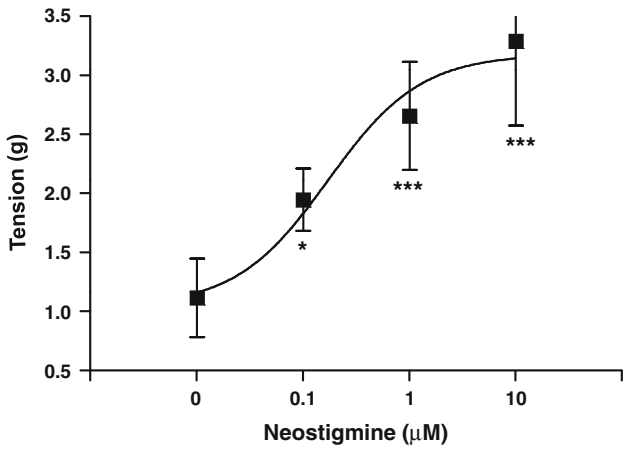


Fig. 6 Effect of neostigmine on 5-Hz c-EFS-induced contraction of rat tracheal rings (mean ± SD, $n = 6$). $^{*}P < 0.05$; $^{***}P < 0.001$ versus neostigmine 0. *c-EFS* continuous electrical field stimulation

of neostigmine. P_{max} was not increased by neostigmine but was increased by application of 2 and 5 Hz c-ES.

Discussion

In this in vitro study with rat tracheal rings we observed that tracheal smooth muscle tension is more responsive to c-EFS than to ordinary EFS for 10 s, and that c-EFS-induced contraction is inhibited by TTX and 4-DAMP whereas it is potentiated by neostigmine.

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

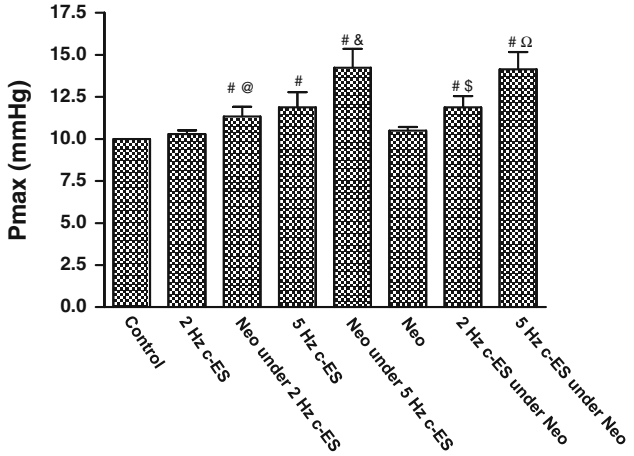


Fig. 7 (1) The effects of 2-Hz c-ES and neostigmine under 2-Hz c-ES on maximum airway pressure (P_{max}) in rats (mean ± SD, $n = 6$). P_{max} was not increased by 2-Hz c-ES but was increased by addition of neostigmine. (2) The effects of 5-Hz c-ES and neostigmine under 5-Hz c-ES on P_{max} in rats (mean ± SD, $n = 6$). P_{max} 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 P_{max} in rats (mean ± SD, $n = 8$). P_{max} was not increased by neostigmine but was increased by addition of 2-Hz c-ES. (4) The effects of neostigmine and 5-Hz c-ES under addition of neostigmine on P_{max} in rats (mean ± SD, $n = 7$). P_{max} was not increased by neostigmine but was increased by addition of 5-Hz c-ES. $^{\#}P < 0.001$ versus control. $^{@}P < 0.001$ versus 2-Hz c-ES. $^{\&}P < 0.001$ versus 5-Hz c-ES. $^{\$}P < 0.$ 01 versus Neo. $^{\Omega}P < 0.001$ versus Neo. *c-ES* continuous electrical stimulation, *Neo* neostigmine

Parasympathetic postganglionic neurons are considered to be close to the targeted end-organ. The tracheal rings used in this 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 and Hatano [7] reported for the isolated trachea that because the ordinary EFS-induced response was abolished by TTX 0.1 μM, cholinergic nerves would mediate the contractile response to ordinary EFS. In this study, c-EFS-induced contraction was blocked completely by TTX, 0.1 μM, suggesting that c-EFS-induced contraction could be mediated by the 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]. Muscarinic M_3 receptors are present on airway smooth muscle cell membranes. Stimulation of M_3 receptors induces airway smooth muscle contraction. In this 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 this study the contractile response to c-EFS would be mediated via muscarinic M_3 receptors.

When the contraction strengths were compared at 5 Hz, c-EFS-induced contraction was 4 times greater than the contraction induced by10-s EFS, and c-EFS-induced contraction was maintained for 3 h. Kirkpatrick and Rooney [10] reported that when tracheal muscle strips were stimulated electrically by supramaximum pulses of 50 Hz, contractile responses continued for 1 h, and the contraction was well maintained during the period of ES. Our current results suggest that when EFS is applied continuously to cholinergic nerves, contraction is enhanced even at low frequency and can be lasting, and yet significant depletion of ACh stores would not take place during c-EFS.

In our in vivo study, P_{max} was not increased by neostigmine. In our previous in vitro study, even the maximum concentration of edrophonium, an anticholinesterase drug, did not cause a contractile response [11]. The lack of effect of edrophonium suggests 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 this in vivo study.

P_{max} was not increased by 2-Hz c-ES of the vagal nerve but was increased by addition of neostigmine. The mechanism involved in the enhancing effect of neostigmine on the c-ES-induced P_{max} 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 to 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 enough ACh to induce bronchoconstriction. However, in the presence of neostigmine, ACh accumulates at the endplate of airway smooth muscle, resulting in the increase in P_{max}.

The dose of neostigmine used in this in vivo study is clinically relevant. Hazizaj and Hatija [12] reported a case of bronchospasm caused by neostigmine. Gouge et al. [13] gave pyridostigmine, an anti-cholinesterase drug, to asthmatics and found no changes in forced vital capacity in any of the patients 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 a second would be enough to excite the cholinergic nerve to cause airway constriction.

Asthma attack is occasionally encountered when neostigmine is used in patients with asthma. Because sugammadex can now be used in clinical setting, 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. P_{max} is increased by c-ES of vagal nerve, and is potentiated by neostigmine. These results suggest that the increased activity of the cholinergic nerve is involved in asthma attack.

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