EFFECTS OF EXPECTORANTS ON THE CANINE TRACHEAL CILIATED CELLS

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Abstract—Many expectorants are clinically effective as they reduce viscosity and facilitate expectoration. There are, however, few reports on effects of expectorants on tracheal ciliated cells. We investigated the effects of N-acetylcysteine, ethylcysteine and bromhexine on the ciliary activities of the canine trachea. Ciliary movement was estimated using a photo-transistor, and intracellular electrical activity was measured with a micro-electrode method, in vitro. N-Acetylcysteine, ethylcysteine and bromhexine in low concentrations under a low perfusion rate (0.1 ml/min) produced an increase in the amplitude and frequency of ciliary beating, while N-acetylcysteine and ethylcysteine caused a cilio-depression in high concentrations. N-Acetylcysteine, ethylcysteine and bromhexine, under a low perfusion rate, did not affect the intracellular electrical activity. On the other hand, these three drugs under a high perfusion rate (1 ml/min) produced no change in the ciliary movement and the intracellular electrical activity in concentrations of $10^{-8}$ to $10^{-4}$ M. These results suggest that the increase in ciliary activity produced by the mucolytic drugs is not due to a direct effect on the ciliated cells, but rather to a mucolytic effect on the mucus around the cilia.

The mucociliary transport system in the respiratory tract plays an important role in the excretion of foreign materials inspired from the atmosphere and sputum originating in the airway. Expectorants including mucolytic drugs have been used widely in the treatment of bronchitis and other obstructive pulmonary diseases. The rationale for their usage is based on their ability to decrease the viscosity of mucus (1, 2) as well as their ability to stimulate mucus production (3). Mucolytic drugs undoubtedly change the rheologic property of respiratory tract secretions (2, 4). In addition, ciliary movement also effectively facilitates expectoration of foreign materials and sputum. Whether or not effects of expectorants on the ciliary transport system are responsible for beneficial effects in patients with obstructive pulmonary diseases remains to be determined.

We studied the action of expectorants (mucolytics) on the ciliary activity using microelectrode and photoelectrical methods.

MATERIALS AND METHODS

Mucous membrane preparation: Male mongrel dogs weighing between 8 and 12 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.) and exsanguinated. A segment of the trachea was excised after a midline incision. The mucous membrane preparation was prepared by removing the smooth muscle layer from the specimen in order to prevent any influence of a smooth
muscular contraction or relaxation. A 5×5 mm piece of the mucous membrane preparation of the tracheal wall was placed on the edge of a glass plate (0.15 mm thick) by a pair of hooks, as shown in Fig. 1, and incubated in Hanks' solution (136.9 mM NaCl, 5.3 mM KCl, 1.1 mM CaCl₂, 0.8 mM MgSO₄, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃ and 11.1 mM glucose) in a 2 ml chamber. The solution used in the experiment was adjusted to pH 7.2±0.1 and continuously oxygenated with a 95% O₂–5% CO₂ mixture. The temperature of the solution was kept at 36.0±0.1°C.

The flow rate of Hanks' solution in the experimental chamber was kept constant (1 ml/min or 0.1 ml/min) using a perfusion pump (Tokyo Rika Kikai, MP-201). At a perfusion rate of 1 ml/min, mucus was taken off from the mucous membrane by the flow and, thus, a mucus-free preparation was available. On the other hand, at a perfusion rate of 0.1 ml/min, mucus produced by the membrane tissue remained around the ciliated membrane (light microscopic observations).

**Ciliary beat recording:** The principle of technique for recording the ciliary beating is shown in Figs. 1 and 2. A microscope objective (water immersion) was focused on the part of the specimen on the edge of glass plate in the chamber. The image of cilia was projected onto a screen bearing an aperture. Changes in the image of cilia were detected with a phototransistor (Sharp, PT500FA) positioned behind the aperture, and were recorded on a pen recorder (Nihon Kohden, WI-640G).

**Membrane potential recording:** The procedure used to record membrane potential was previously described (5). The experiment was carried out under a double shielded condition. A microelectrode (the outer diameter of the tip being less than 0.5 μm and the resistance being 30–60 MΩ) filled with 3 M KCl solution, was put into an appropriate holder and mounted on a micromanipulator. The potential difference between the microelectrode inside the tracheal epithelium and the indifferent electrode (Ag-AgCl) placed in the medium was measured by using a high input impedance amplifier (Nihon Kohden, WI-640G).

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Fig. 1. Schematic diagram of the experimental apparatus. The specimen is placed on a glass plate using a platinum clip. Test solutions are perfused through the experimental chamber at a constant rate of 1 ml/min or 0.1 ml/min.
MZ-4) (Fig. 2). All measurements of the potentials were displayed on an oscilloscope (Nihon Kohden, VC-7) and recorded on a pen recorder (Nihon Kohden, WI-640G). The impalement of an electrode was performed by operating the manipulator gently by 1–2 μm. When the electrode touched the cell membrane, an abrupt change in the tip potential occurred and was audible with a loudspeaker.

Calculation of data: The ciliary beating and the intracellular potential were recorded for 2 sec at every 30 sec during 5 min after a start of drug administration. The changes in frequencies of ciliary beating and oscillatory potentials were expressed as mean % change±S.E. The percent changes were calculated by comparing the frequency or amplitude during a 5 min-period of drug treatment with the frequency or amplitude during a 5 min-period before the treatment. The Student’s unpaired t-test was used for the statistical analysis of the data.

Drugs: Acetylcholine chloride (Tokyo Chemical Co.), potassium chloride (Wako Pure Chemical Co.), N-acetyl-L-cysteine (Showa Chemical Co.), L-cysteine ethylester hydrochloride (Yoshitomi) and bromhexine hydrochloride (Boehringer) were dissolved in Hanks’ solution.

RESULTS

Effects of acetylcholine and potassium ion on the ciliary activity: Penetration of a microelectrode into a ciliated cell at a depth of 5–25 μm from the mucosal surface revealed the membrane potential to be −28.8±1.8 (S.E.) mV (N=42). The amplitude and frequency of oscillatory potentials were 0.22±0.03 mV and 13.1±0.9 Hz, respectively, under a perfusion rate of 1 ml/min. Almost the same values were obtained under a rate of 0.1 ml/min.

The beating frequency was stable at least for one hour. In Fig. 3 are shown frequencies of ciliary beating under perfusions at constant rates of 1 ml/min and 0.1 ml/min. The average frequencies at perfusion rates of
1 ml/min and 0.1 ml/min were 12.9±1.1 and 15.9±0.7 beats/sec, respectively. The beat frequency at 1 ml/min was about 20% less than that at 0.1 ml/min. When Hanks’ solution was perfused again at 0.1 ml/min after an 1 hr-perfusion at 1 ml/min, the beat frequency increased to about 16 beats/sec 30 min after the change in perfusion rate.

When Hanks’ solution containing 10^{-6} M acetylcholine was introduced into the

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**Fig. 3.** Ciliary beating of canine tracheal ciliated cells under a perfusion rate of 1 ml/min and 0.1 ml/min (control Hanks’ solution). A, The mean frequencies at perfusion rates of 1 ml/min (△) and 0.1 ml/min (○) for 30 min were 12.9±1.1 and 15.9±0.7 beats/sec, respectively. Each point represents the mean with S.E. for 5 experiments. B, Typical ciliary beating patterns at perfusion rates of 1 ml/min and 0.1 ml/min.

**Fig. 4.** Effects of acetylcholine and potassium ion on the ciliary beating of canine tracheal ciliated cells at a perfusion rate of 1 ml/min. Acetylcholine (10^{-6} M) increased the amplitude and frequency of ciliary beating, while KCl (8×10^{-2} M) ceased the beating.
chamber at 1 ml/min, the ciliary movement was activated to a maximum plateau in beating frequency within 3 min (Fig. 4). The enhanced ciliary activity decreased to the control level after the acetylcholine solution was displaced by control Hanks’ solution. When Hanks’ solution containing 8×10⁻² M KCl was perfused at 1 ml/min, the ciliary beating gradually decreased and finally ceased (Fig. 4).

Effects of N-acetylcysteine, ethylcysteine and bromhexine under a high perfusion rate: When Hanks’ solution containing N-acetylcysteine or ethylcysteine (10⁻⁸–10⁻⁴ M) was introduced into the chamber at 1 ml/min for 30 min, the ciliary movement was unaltered (Fig. 5). An exposure to bromhexine (10⁻⁸–10⁻⁴ M) for 30 min also produced no change in the frequency and amplitude of beating. In a concentration of 10⁻³ M of N-acetylcysteine and ethylcysteine, the ciliary beat frequency showed a slight decrease. N-Acetylcysteine, ethylcysteine and bromhexine did not affect the intracellular electrical activity (Fig. 5).

Effect of N-acetylcysteine under a low perfusion rate: When Hanks’ solution containing N-acetylcysteine was perfused at 0.1 ml/min, the ciliary movement was activated. An increase in ciliary beat amplitude was seen in concentrations of 10⁻⁷ and 10⁻⁶ M. On the other hand, reductions in the ciliary beat frequency and amplitude were seen in 10⁻⁴ and 10⁻³ M (Fig. 6). N-Acetylcysteine produced no change in the amplitude and frequency of oscillatory potentials up to a concentration of 10⁻⁴ M. In 10⁻³ M of this agent, the amplitude and frequency of oscillatory potentials decreased. The depressive effect of N-acetylcysteine disappeared by washing with control Hanks solution for 30 min.

Effect of ethylcysteine under a low perfusion rate: When Hanks solution containing 10⁻⁷–10⁻⁵ M of ethylcysteine was perfused at 0.1 ml/min, increases in the amplitude and frequency of ciliary beating were seen (Fig. 7). In concentrations of 10⁻⁴ M or above, there was a significant decrease in the amplitude and frequency

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**Fig. 5.** Effects of N-acetylcysteine, ethylcysteine and bromhexine on (A) the ciliary beating frequency and (B) the oscillatory potentials of canine tracheal ciliated cells at a perfusion rate of 1 ml/min. Each point represents the mean for 5 experiments.
Ciliary beat frequency depends to a large extent on viscosity of the gel layer and weight of the overlying mucus (6–8). It is pointed out that the cilia normally stand in a non-viscous interciliary sol layer and that the viscous mucus over the sol layer is transported by the tips of cilia which barely project out the interciliary sol layer. An increase or decrease in the viscosity of mucus would therefore affect the mucus transport. The present finding indicates that ciliary activity is increased in a concentration range of $10^{-7}$–$10^{-5}$ M of N-acetylcysteine and ethylcysteine under a high perfusion rate. The effect is probably due to a moderate lysis of the mucus around the cilia, which leads to a more effective transport of mucus. This assumption is supported by the observation that in patients with chronic bronchitis the expectoration was facilitated through a lysis of the mucus by N-acetylcysteine (9). N-Acetylcysteine and ethylcysteine are believed to exert the actions by disruption of ciliary beating. Ethylcysteine in $10^{-3}$ M caused decreases in the amplitude and frequency of oscillatory potentials, which recovered to control level after a removal of the agent.

**Effect of bromhexine under a low perfusion rate:** Bromhexine was perfused at a constant rate of 0.1 ml/min. Under this condition, the amplitude and frequency of ciliary beating increased significantly in concentrations of $10^{-7}$–$10^{-5}$ M (Fig. 8). The increase in the frequency of ciliary beating appeared within 5 min after treatment with bromhexine, while the increase in the amplitude occurred after 10 min. Bromhexine, however, caused no change in the amplitude and frequency of oscillatory potentials.

**DISCUSSION**

Ciliary beat frequency depends to a large extent on viscosity of the gel layer and weight of the overlying mucus (6–8). It is pointed out that the cilia normally stand in a non-viscous interciliary sol layer and that the viscous mucus over the sol layer is transported by the tips of cilia which barely project out the interciliary sol layer. An increase or decrease in the viscosity of mucus would therefore affect the mucus transport. The present finding indicates that ciliary activity is increased in a concentration range of $10^{-7}$–$10^{-5}$ M of N-acetylcysteine and ethylcysteine under a high perfusion rate. The effect is probably due to a moderate lysis of the mucus around the cilia, which leads to a more effective transport of mucus. This assumption is supported by the observation that in patients with chronic bronchitis the expectoration was facilitated through a lysis of the mucus by N-acetylcysteine (9). N-Acetylcysteine and ethylcysteine are believed to exert the actions by disruption of...
Fig. 7. Effects of ethylcysteine on (A) the ciliary beating and (B) the oscillatory potentials of 0.1 ml/min. Other explanations as in Fig. 6.

Fig. 8. Effects of bromhexine on (A) the ciliary beating and (B) the oscillatory potentials of canine tracheal ciliated cells at a perfusion rate of 0.1 ml/min. Other explanations as in Fig. 6.
disulfide bonds in mucoprotein molecules through their free sulfhydryl groups, thereby reducing the viscosity of mucus (10).

In a low concentration of N-acetylcysteine, an increase in mucus production was verified in a morphological study by Iravani et al. (11). In their experiment, both increases in depth of mucus layer and in numbers of endoplasmic reticula and Golgi complexes were observed, which indicate an increased function of the mucus secreting apparatus.

The present experiment showed that N-acetylcysteine and ethylcysteine caused a cilio-depression in concentrations of $10^{-4}$ M and above. A reduction in ciliary activity after treatments with these drugs may result from a rheological change in mucus such as an excessive reduction in the viscosity and elasticity of mucus. This is supported by a finding that ciliary beat frequency is dependent on elasticity, adhesiveness and quantity of the overlying mucus (12).

N-Acetylcysteine reportedly induces a partially reversible ciliostasis of the tracheal epithelium in concentrations of 0.3 M and above (13, 14).

Bromhexine increased the frequency and amplitude of ciliary beating under a low perfusion rate. The viscosity of mucoid sputum is reportedly decreased by bromhexine through a depolymerization of high molecular weight mucoprotein fibers (15-17). Electron microscopical studies suggested that the agent may act within the mucus-secreting cells (18). Bromhexine increases the volume and reduces the viscosity of bronchial secretions in laboratory animals (3, 19).

A bromhexine metabolite had two effects on mucus production (3). The first effect occurred immediately after application of the drug and the second developed over the course of 10–16 min. This suggests that the immediate effect of the drug would be a discharge of the already formed mucus granules and the late effect would be a stimulation of new mucus production. Therefore, the facilitation of the amplitude and frequency of ciliary beating caused by bromhexine may result from both a lysis of mucus and an increase in mucus production.

Acetylcholine concomitantly increased the frequencies of both ciliary movement and oscillatory potentials. KCl induced a decrease in frequencies of both ciliary movement and oscillatory potentials (present data, 5). On the other hand, the frequency and amplitude of oscillatory potentials showed no change after treatment with the mucolytic drugs used in our study. Therefore, the enhanced ciliary beating induced by N-acetylcysteine, ethylcysteine and bromhexine cannot be ascribed to direct actions on the ciliated cells, but rather to an indirect action by changing the amount and rheology of the mucus. With the micro-electrode and photoelectrical methods we used, it is possible to analyse both direct effects of drugs on ciliated cells and rheological factor of total mucociliary system. We consider that the intracellular electrical activity (oscillatory potentials) indicates the activity of a cell itself, independent of the mucus rheology.

We conclude that the expectorants used in this study indirectly affect the ciliary movement through mucolytic action. Also, the present method proved to be useful for studying the mechanisms of action of drugs on the tracheal ciliated cells.

REFERENCES


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