Eur Respir J, 1996, 9, 1632–1636
DOI: 10.1183/09031936.96.09081632
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European Respiratory Journal
ISSN 0903 - 1936

Cholinergic control of rabbit tracheal transepithelial potential difference *in vivo*

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Cholinergic control of rabbit tracheal transepithelial potential difference in vivo. *J. Tamaoki, A. Chiyotani, E. Tagaya, H. Takemura, K. Konno. ©ERS Journals Ltd 1996.*

ABSTRACT: The aim of the present study was to investigate the role of the autonomic nervous system in the regulation of airway epithelial ion transport *in vivo*.

Rabbits were anaesthetized and mechanically-ventilated through a cannula inserted above the carina. The upper tracheal mucosa was exposed, and the electrical potential difference (PD) between the mucosal surface and the submucosal space was continuously measured by a high-impedance voltmeter under open-circuit conditions.

Perfusion of the mucosa with atropine caused a rapid decline in PD from -20.1±2.0 to -15.2±0.9 mV ($p<0.01$), whereas phentolamine, propranolol, or the tachykinin antagonist, FK224, had no effect. Cutting both cervical vagus nerves decreased PD to the same degree as did atropine. Exogenously applied acetylcholine increased PD in a dose-dependent manner. Topical application of ipratropium bromide reduced the baseline value PD in a dose-dependent manner. The maximal decrease in PD was 4.3±0.3 mV ($p<0.01$), and the dose required to produce a half-maximal effect was 34 µg. Perfusion with either amiloride, a Na channel blocker, and diphenylamine-2-carboxylate, a Cl channel blocker, decreased the baseline PD, and the subsequent application of ipratropium bromide further decreased the PD in each case.

We conclude that a cholinergic neural component may play a role in the generation of tracheal potential difference *in vivo*, probably involving stimulation by endogenously released acetylcholine of both Cl secretion and Na absorption across the airway epithelium.

Eur Respir J., 1996, 9, 1632–1636.

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Keywords: Acetylcholine
airway epithelium
ion transport
ipratropium bromide

Received: September 1 1995
Accepted after revision February 22 1996

This work was supported in part by a grant for Scientific Research No. 06670632 from the Ministry of Education, Science and Culture, Japan.

It has been generally accepted that the amount and physicochemical properties of airway surface fluid can be influenced by the ion transport function of airway epithelium [1]. Epithelial cells in the central airway absorb Na from and secrete Cl toward the lumen, and the net ion flux through epithelial cellular and paracellular paths generates a transepithelial electrical potential difference (PD), which concomitantly promotes water movement across the airway mucosa [2]. Although the regulation of airway epithelial bioelectric properties has been extensively studied in excised tracheal sheets and cultured cells [3, 4], these *in vitro* findings may not necessarily reflect ion transport *in vivo* because of the lack of innervation and blood supply. For example, previous *in vitro* experiments have shown that exogenously applied autonomic neurotransmitters, including catecholamines and acetylcholine, increased PD or short-circuit current of tracheal epithelial cells [5, 6], but application of autonomic receptor antagonists *per se* had no effect [7]. The latter finding could be due to the lack of endogenous neurotransmitters in the *in vitro* experimental system, and it remains unknown whether the autonomic nervous system is involved in the regulation of airway epithelial ion transport *in vivo*.

Inhaled anticholinergic drugs are widely-used in the treatment of chronic obstructive disease and asthma. We have recently shown that inhalation of oxitropium bromide can reduce sputum production in patients with chronic obstructive pulmonary diseases, probably through an inhibition both of mucus glycoprotein secretion from submucosal glands and water transport by airway epithelial cells toward the respiratory lumen [8]; indicating that endogenous acetylcholine is possibly operating in the epithelial ion transport process. Therefore, in the present study, to determine the regulatory role of cholinergic nerves in airway epithelial ion transport *in vivo*, we measured tracheal mucosal PD of anaesthetized rabbits under open-circuit conditions.

Materials and methods

Measurement of PD

Measurement of tracheal mucosal PD *in vivo* has been described in detail previously [9, 10]. Briefly, Japanese white male rabbits, weighing 1.8–2.5 kg, were anaesthetized with intraperitoneal α-chloralose (50 $mg \cdot kg^{-1}$) and urethane (500 $mg \cdot kg^{-1}$), and the trachea was exposed. A polyethylene tube was inserted into the trachea 5 mm above the carina, through which the respirator (model SN-480-7; Shinano Co., Tokyo, Japan) was connected and mechanical ventilation was performed (tidal volume (V_T) 10 $ml \cdot kg^{-1}$, respiratory rate (f_R) 60 $breaths \cdot min^{-1}$). The upper tracheal cartilage rings were then incised

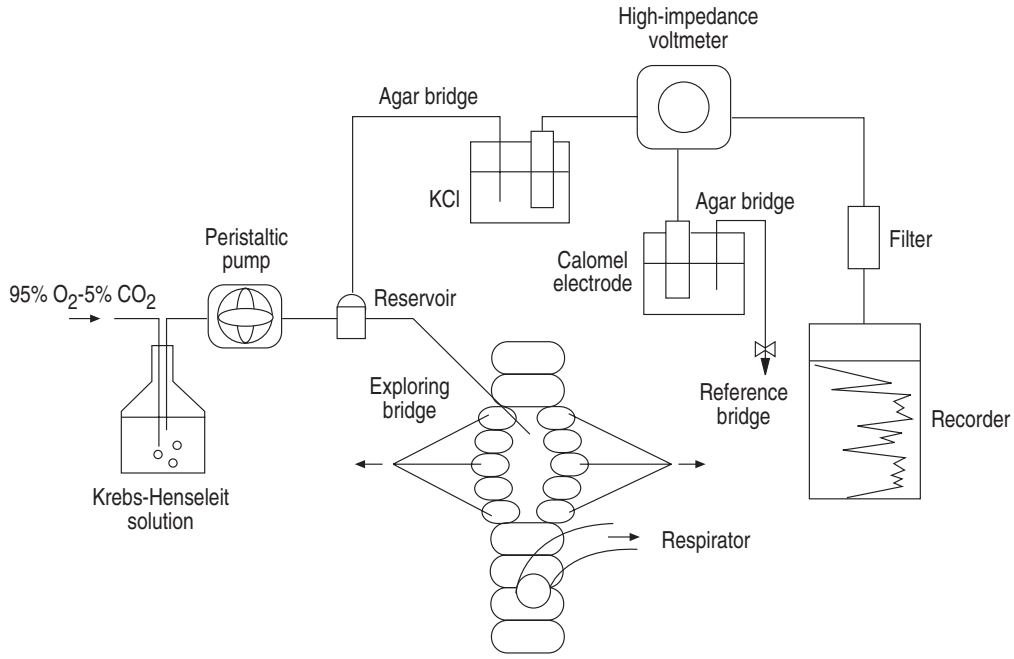

Fig. 1. – Schematic diagram for the measurement of transepithelial potential difference across rabbit tracheal mucosa *in vivo*. An exploring bridge was placed on the surface of posterior membrane of the trachea and a reference bridge was inserted into the subcutaneous space of right anterior chest.

transaxially and the surface of membranous portion was exposed (fig. 1).

The exploring bridge constructed of polyethylene tube (2.5 mm diam) for *in vivo* measurement of airway epithelial PD was placed on the surface of the posterior membrane, 15 mm above the carina. Contact with the tracheal mucosal surface was ensured by continuous perfusion (0.3 $mL \cdot min^{-1}$) through the bridge with Krebs-Henseleit (K-H) solution, of the following composition: 118 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 1.2 mM $NaHCO_3$, and 25.5 mM glucose, warmed at 37°C and adjusted pH to 7.4. The whole area of exposed mucosal surface of the trachea (approximately 1 cm^2) was perfused, and the perfusate was spontaneously removed by overflow. The perfusion reservoir was connected to the calomel electrode *via* a polyethylene tube (1.5 mm diameter) filled with 3% agar in saline.

The reference bridge, a 21-gauge needle that contained 3% agar in saline, was inserted into the subcutaneous space of the right anterior chest wall, which was isoelectric with the adventitial surface of the trachea [10]. Each bridge was connected by a calomel electrode to a high-impedance voltmeter (model CEZ-9100; Nihon Kohden, Tokyo). The electrical signal was filtered to remove 60-cycle interference, and electrical PD between the tracheal mucosal surface and the subcutaneous space was continuously recorded as transmembrane PD on a pen recorder (model SR 6335; Graphtec, Tokyo).

Protocol

To determine the involvement of autonomic neurotransmitters in the generation of tracheal PD, the tracheal mucosa was continuously perfused with K-H solution and, when the PD became stable, the following drugs were sequentially added to the superfusing solution in random order: phentolamine (10^{-5} M), an α-adrenergic receptor antagonist; propranolol (10^{-5} M), a β-adrenergic receptor antagonist; atropine (10^{-5} M), a cholinergic muscarinic receptor antagonist; and FK224 (10^{-5} M), an antagonist for tachykinin neurokinin-1 and -2 (NK_1 and NK_2) receptors [11]. The tracheal mucosal surface was perfused with an antagonist for 10 min. The mucosa was then washed with K-H solution for 15 min, and the next antagonist was added to the solution. Among the autonomic antagonists used, only atropine caused a decrease in PD, and this effect was not influenced by the sequence of perfusion. In a control experiment, the effect of the vehicle of the drugs (sterile saline) alone was determined.

To further assess the contribution of the parasympathetic neural pathway, the cervical vagus nerves were dissected and cut bilaterally, while the tracheal PD was monitored. In addition, to confirm whether acetylcholine was actually capable of increasing the PD, acetylcholine (10^{-8} to 10^{-4} M) was added to the superfusate. In this experiment, acetylcholine was cumulatively given at 5 min intervals or 1 min after the stable plateau was achieved, whichever was the longer period, while the response to each dose was determined.

To examine the effect of an inhaled antimuscarinic agent on tracheal PD, ipratropium bromide (20–100 μg) was applied directly to the mucosal surface using a metered-dose inhaler, in a cumulative manner with the same time sequence as the experiment with acetylcholine. A laboratory-made adaptor, with an outflow orifice 8 mm in diameter, was attached to the inhaler, and ipratropium bromide was applied to the mucosal surface through the adaptor. To assess whether the response of PD to ipratropium bromide was due to an alteration in Na absorption from the airway lumen or Cl secretion toward the submucosa across the airway epithelium, the mucosa was perfused with K-H solution containing amiloride (10^{-4} M), a Na channel blocker [12], or diphenylamine-2-carboxylate (10^{-4} M), a Cl channel blocker [13], and 5 min later ipratropium bromide at 60 μg was

applied. In addition, to confirm whether acetylcholine actually alters movement of Na and Cl, the effects of amiloride and diphenylamine-2-carboxylate on acetylcholine (10^{-4} M)-induced increase in PD were likewise determined.

Drugs

The following drugs were used: phentolamine hydrochloride, DL-propranolol hydrochloride, atropine sulphate, acetylcholine chloride, amiloride (Sigma Chemical Co., St. Louis, MO, USA); diphenylamine-2-carboxylate (Nacalai Tesque, Kyoto, Japan). FK224 was a gift from Fujisawa Pharmaceuticals, Osaka, Japan. Ipratropium bromide was a gift from Teijin Co., Tokyo. Amiloride was first dissolved in distilled water and subsequently diluted with K-H solution, and other agents were directly dissolved in K-H solution.

Statistics

All data are expressed as mean±SEM. Statistical analysis was performed by analysis of variance (ANOVA) and Fisher's multiple range tests, and a p-value of less than 0.05 was considered to be statistically significant.

Results

The *in vivo* PD of rabbit tracheal mucosa became stable within 5 min after perfusion with K-H solution, and the baseline PD value was -19.4±1.7 mV (n=38), lumen negative. As shown in figure 2, addition of atropine to K-H solution superfusing the tracheal mucosa decreased PD from -20.1±2.0 to -15.2±0.9 mV ($p<0.01$; n=9) within 1 min, whereas the vehicle (saline) alone had no effect, with the change from the baseline PD being less than 0.6 mV throughout the experiment. The inhibitory effect of atropine on the PD was completely reversed within 15 min by the subsequent perfusion of the mucosa with fresh K-H solution. In contrast, application of phentolamine, propranolol, or FK224 did not alter the baseline PD. Bilateral section of the cervical vagus nerves decreased the PD to -14.7±1.3 mV ($p<0.01$; n=9), a value not significantly different from the PD in the presence of atropine.

Addition of acetylcholine to the superfusate rapidly increased the baseline PD. This effect was dose-dependent (fig. 3), the maximal increase and the acetylcholine concentration required to produce a half-maximal effect (EC_{50}) being 5.6±0.8 mV ($p<0.01$; n=7) and 0.7 µM, respectively.

Topical application of ipratropium bromide to the tracheal mucosal surface by a metered-dose inhaler decreased the PD in a dose-dependent manner (fig. 4): the maximal decrease from the baseline value was 4.3±0.3 mV ($p<0.01$; n=10) observed at 60 µg ipratropium bromide,

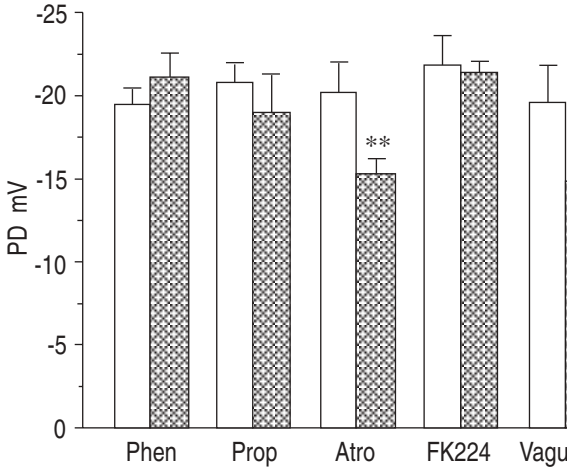

Fig. 2. – Effects of pharmacological blocking agents and cutting of bilateral cervical vagus nerves on rabbit tracheal potential difference (PD). After obtaining baseline PD ( ), phentolamine (Phen) 10^{-5} M; propranolol (Prop) 10^{-5} M, atropine (Atro) 10^{-5} M, or FK224 10^{-5} M, was added to the superfusing solution, or the cervical vagus nerves were cut (postintervention PD). Data are presented as mean±SEM; n=9 for each column. **: $p<0.01$, significantly different from the baseline value.

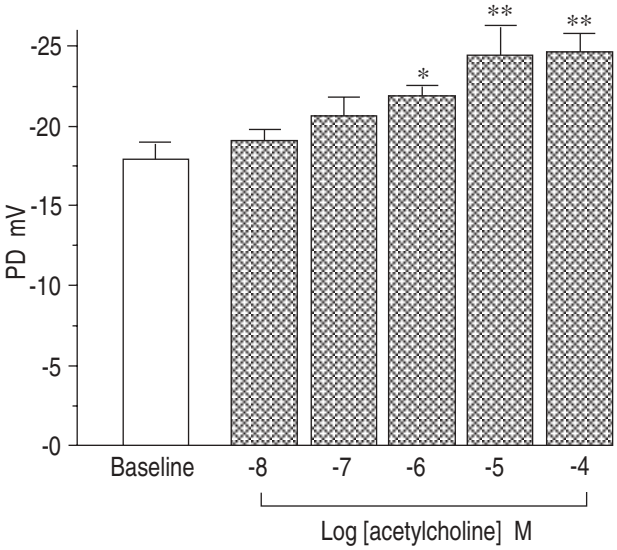

Fig. 3. – Concentration-dependent effect of acetylcholine on rabbit tracheal potential difference (PD). After obtaining baseline PD ( ), acetylcholine was added to the superfusate, while the response to each concentration was determined ( ). Data are presented as mean±SEM; n=7 for each column. *: $p<0.05$; **: $p<0.01$, significantly different from the baseline value.

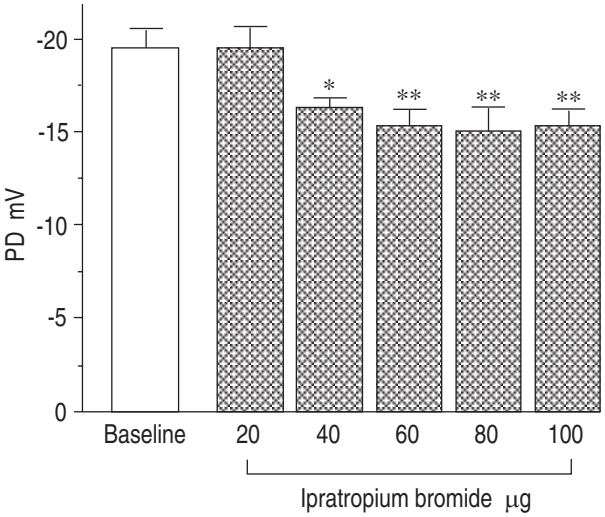

Fig. 4. – Dose-dependent effect of ipratropium bromide on rabbit tracheal potential difference (PD). After obtaining baseline PD ( ), ipratropium bromide was applied directly to the mucosal surface in a cumulative manner, while the response to each dose was determined ( ). Data are presented as mean±SEM; n=10 for each column. *: $p<0.05$; **: $p<0.001$, significantly different from the baseline value.

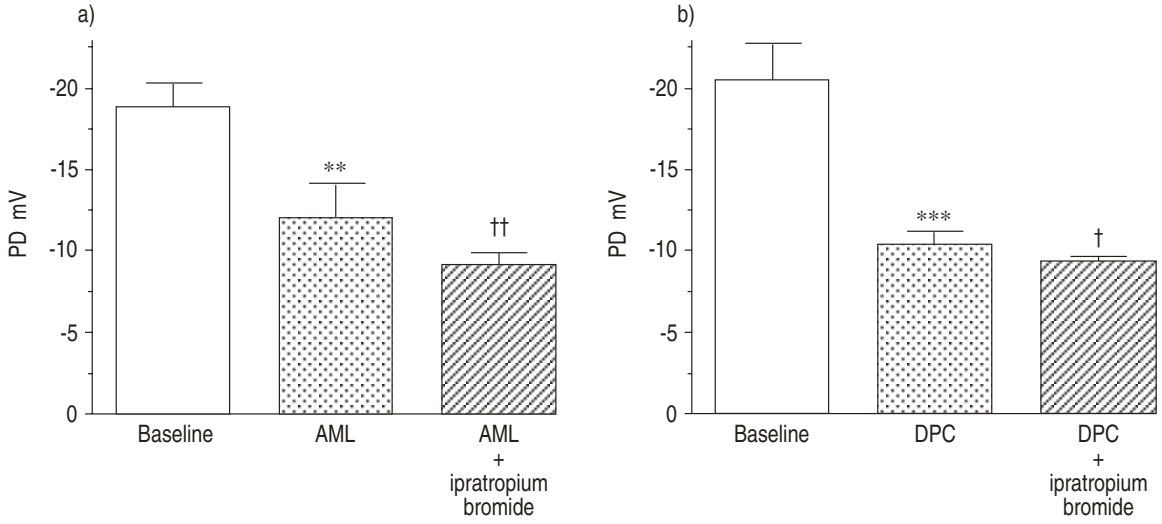

Fig. 5. – Effects of ipratropium bromide on rabbit tracheal potential difference (PD) in the presence of ion channel blockers. After obtaining baseline PD, tracheal mucosa was perfused with: a) amiloride (AML), 10^{-4} M: or b) diphenylamine-2-carboxylate (DPC), 10^{-4} M, and ipratropium bromide at 60 µg was then applied. Data are presented as mean±SEM; n=8 for each column. **: p<0.01; ***: p<0.001, significantly different from the corresponding baseline values; †: p<0.05; ††: p<0.01, significantly different from the response to the ion channel blocker alone.

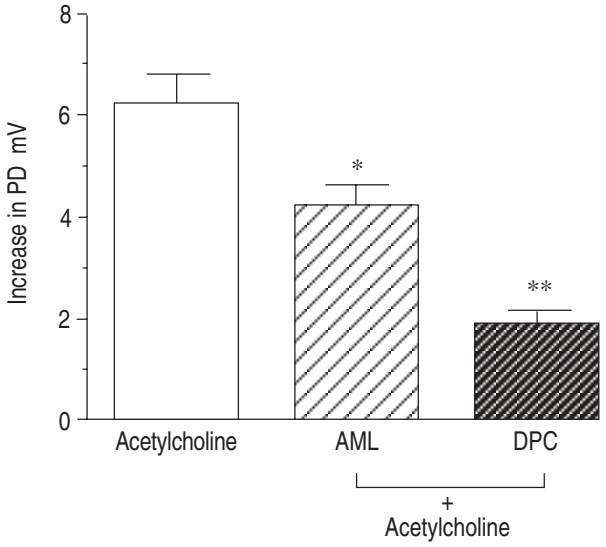

Fig. 6. – Effects of amiloride (AML), 10^{-4} M, and diphenylamine-2-carboxylate (DPC), 10^{-4} M, on the increase in rabbit tracheal potential difference (PD) produced by acetylcholine (10^{-4} M). Data are presented as mean±SEM; n=6 for each column. *: p<0.05; **: p<0.01, significantly different from the response to acetylcholine alone.

and the dose required to produce a half-maximal effect (ED_{50}) was 34 µg (n=10). Perfusion of amiloride-containing K-H solution decreased the tracheal PD from -18.7±1.4 to -12.0±2.2 mV (p<0.01; n=8), and the subsequent application of ipratropium bromide at 60 µg further decreased the PD by 2.9±0.4 mV (p<0.01; n=8) (fig. 5a). Similarly, perfusion of the mucosa with diphenylamine-2-carboxylate decreased the PD from -20.3±2.2 to -10.4± 0.8 mV (p<0.001; n=8), and the remaining PD was further decreased by the subsequent application of 60 µg ipratropium bromide by 1.1±0.5 mV (p<0.05; n=8) (fig. 5b). The ipratropium bromide-induced decrease in PD in the presence of amiloride was, thus, greater than that in the presence of diphenylamine-2-carboxylate (p<0.05).

Addition of acetylcholine (10^{-4} M) to the solution increased PD from -18.3±2.0 to -24.5±2.3 mV (p<0.01; n=6). This increase was inhibited by amiloride and diphenylamine-2-carboxylate by 32±8% (p<0.05; n=6) and 69± 10% (p<0.01; n=6), respectively (fig. 6).

Discussion

Our *in vivo* studies demonstrate that the cholinergic parasympathetic component of the autonomic nervous system plays a role in generating the PD across rabbit tracheal mucosa, which may thus regulate the concomitant transport of electrolytes and water. This conclusion is based on the findings that the baseline value of tracheal PD was substantially decreased either by perfusion of tracheal mucosa with atropine or by direct application of the inhaled anticholinergic agent ipratropium bromide.

There is ample evidence that airway epithelial cells secrete and absorb electrolytes and water across airway mucosa; thereby regulating the output and composition of the respiratory tract secretions [1–3]. Under unstimulated condition, transepithelial PD in the airway mucosa appears to result mainly from secretion of Cl toward the lumen and reabsorption of Na into the interstitium, which consequently generates lumen negative PD [9]. In our experiment, the baseline value of rabbit tracheal *in vivo* PD was slightly smaller than that previously reported [9], and it was approximately twofold greater than that reported in rabbit excised tracheal mucosa *in vitro* [14].

One possible explanation would be that the lower PD *in vitro* could result from the lack of contribution of autonomic neurotransmitters and other endogenous bioactive substances in the circulating blood. Indeed, a previous *in vitro* study showed that application of a combination of autonomic receptor antagonists, including atropine and propranolol, did not affect airway epithelial bioelectric properties [7], but we found that application of atropine or cutting of cervical vagus nerves caused a rapid decrease in PD *in vivo*. Likewise, direct application of ipratropium bromide reduced PD in a dose-dependent fashion. These results indicate that acetylcholine, which is spontaneously released from vagal nerve terminals [15], may be involved in the generation of tracheal PD under baseline

condition. Furthermore, exogenously applied acetylcholine caused an increase in PD, indicating that acetylcholine is actually capable of stimulating transtracheal ion transport function *in vivo*. In contrast, a previous study on canine trachea by BOUCHER *et al*. [9] showed that *in vivo* and *in vitro* PDs were similar, acetylcholine did not alter *in vivo* PD, and that atropine reduced *in vivo* PD only at a high concentration (10^{-3} M). This difference may be explained by the species or by other, undefined, experimental details.

It has been known that a variety of airway functions are controlled by cholinergic, adrenergic, and nonadrenergic noncholinergic components of the autonomic nervous system [16, 17]. In the present study, in contrast to the effects of anticholinergic agents, application of the adrenergic receptor antagonists, phentolamine and propranolol, or the tachykinin NK_1 and NK_2 receptor antagonist FK224 [11] had no effect on PD, suggesting that the involvement of catecholamines released from adrenergic nerves and in the circulating blood, or tachykinins released from capsaicin-sensitive sensory nerves seems unlikely. This notion can also be supported by the finding that atropine decreased the tracheal PD to the same extent as did vagal nerve cutting.

Perfusion of the tracheal mucosa with the Na channel blocker amiloride [12] decreased the tracheal PD, and the remaining PD may depend largely on Cl secretion toward the respiratory lumen. Similarly, perfusion with the Cl channel blocker diphenylamine-2-carboxylate [13] decreased the PD, and the remaining PD may depend on Na absorption from the lumen toward the submucosa. We found that the subsequent application of ipratropium bromide further reduced the PD under each condition, and the magnitude of the decrease was greater when amiloride was present than when diphenylamine-2-carboxylate was present. These results suggest that endogenous acetylcholine may be stimulating both Cl secretion and Na absorption, and that the former action predominates.

This conclusion is consistent with the previous *in vitro* finding that acetylcholine increases the short-circuit current of the canine tracheal epithelium probably through a stimulation of Cl and Na transport [6]. Furthermore, in the present *in vivo* experiment, we found that diphenylamine-2-carboxylate inhibited the increase in PD produced by exogenously applied acetylcholine to a greater extent than did amiloride, thereby supporting the suggested mechanism of endogenous acetylcholine. However, possible contributions of other ion transport processes, such as amiloride-insensitive Na, Na-glucose cotransport and bicarbonate diffusion, cannot be ruled out [18].

Airway hypersecretion is one of the characteristic features of asthma and chronic obstructive pulmonary disease. A recent report from our laboratory showed that inhalation of the anticholinergic agent, oxitropium bromide, reduces sputum production in man and that this effect may be derived, at least in part, from the inhibition of airway epithelial Cl transport and the concomitant reduction of water secretion toward the respiratory lumen [8]. The results of the present study in the rabbit may, thus, be consistent with the inhibitory effect of anticholinergic agent on airway secretion in man, and suggest that a cholinergic neural pathway may play a role in the regulation of airway epithelial PD and, hence, water transport across the respiratory lumen *in vivo*.

Acknowledgements: The authors thank M. Shino and Y. Sugimura for their technical assistance.

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