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Ambroxol Inhibits Na^+ Absorption by Canine Airway Epithelial Cells in Culture

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Abstract—To study the effect of ambroxol on ion transport functions of airway mucosa, we measured bioelectric properties of canine cultured tracheal epithelium under short-circuit conditions in-vitro. Addition of ambroxol to the submucosal but not to the mucosal solution in an Ussing chamber decreased short-circuit current, transepithelial potential difference and cell conductance. The ambroxol-induced decrease in short-circuit current was not affected by bumetanide or diphenylamine-2-carboxylate, but it was abolished by pretreatment of cells with amiloride. These results suggest that ambroxol may selectively inhibit Na^+ absorption by airway epithelium, thereby increasing water composition in airway surface fluid and reducing mucus viscosity.

Airway epithelial cells play an important role in the regulation of mucociliary clearance whereby cellular debris and inhaled bacteria and particles are removed from the conducting airways of the respiratory tract. It has been known that this transport function seems dependent on the interaction between ciliary motility and airway surface fluid (Wanner 1977), and that the amount and the rheological properties of airway surface fluid can be largely influenced by ion transport processes in airway epithelium (Nadel et al 1985).

Ambroxol, *trans*-4-[(2-amino-3,5-dibromobenzyl) amino] cyclohexanol hydrochloride, a drug used to increase surfactant secretion in the lungs, has been reported to be effective in reducing exacerbation of chronic bronchitis (Donner 1984) and in protecting inflammatory reactions (Stockley et al 1988; Bianchi et al 1990). However, the effects of this agent on airway epithelial functions remain unknown. Therefore, in the present experiment, to determine whether ambroxol alters ion transport and, hence, water movement across airway mucosa, we studied bioelectric properties of canine cultured epithelium under short-circuited conditions in-vitro.

Materials and Methods

Materials

Ambroxol was obtained from Teijin Co. Ltd (Tokyo, Japan), dissolved in dimethylsulphoxide (DMSO, 10^{-2} M) and subsequently diluted in Krebs-Henseleit solution. Bumetanide and amiloride were purchased from Sigma Chemicals (St Louis, MO, USA). Diphenylamine-2-carboxylate (DPC) was purchased from Nakarai Chemicals (Tokyo, Japan).

Cell culture

Mongrel dogs, 19–25 kg, of either sex were anaesthetized with pentobarbitone sodium (35 mg kg^{-1}, i.v.), and their trachea were removed. The resected sections of tracheal mucosa were placed in fresh modified Eagle's medium containing 0·1% proteinase type XIV and maintained at 4°C for 24 h. After mild agitation the tissue sections were removed from the medium and the cells were concentrated by centrifugation (800 *g*). The pellets of epithelial cells were washed twice with modified Eagle's medium containing 10% foetal calf serum to neutralize the proteinase. These cells were suspended in Ham's nutrient F12 medium containing 0·3% foetal calf serum, 5 $\mu g\ mL^{-1}$ insulin, 5 $\mu g\ mL^{-1}$ transferrin and 10 $\mu g\ mL^{-1}$ epidermal growth factor, plated at a density of $1{\cdot}5 \times 10^6\ cm^2$ using 1 mL of Ham's nutrient F12 medium per Linbro tissue culture multi-well plate (Flow Lab Inc., McLean, VA, USA), and grown on nucleopore polycarbonate filters (13 mm diam., 0·45 μm pore size) at 37°C in a CO_2 incubator. On the seventh day of incubation, cells became confluent and were used for the measurement of bioelectric properties.

Measurement of bioelectric properties

The filters on which tracheal epithelial cells were grown were mounted in an Ussing chamber (surface area, 0·5 cm^2) bathed with Krebs-Henseleit solution maintained at 37°C and bubbled with 95% O_2–5% CO_2 (Leikauf et al 1985). The calomel half-cells were paired to within 0·2 mV of each other. The potential difference across the epithelium was measured through two polyethylene bridges containing 3% agar in 1 M KCl, located 1 mm from each side of the epithelial surface and connected to a calomel half-cell and a high-impedance voltmeter. Another pair of polyethylene bridges containing 3% agar in 0·9% NaCl (saline) located 10 mm from the tissue was used to pass sufficient current through the chamber and the cells to bring the transepithelial potential difference to zero. This short-circuit current was recorded continuously except for 3 s every 10 min when the voltage clamp was turned off and the potential difference was recorded. Tissue conductance in ms cm^{-2} was calculated by dividing the measured short circuit current per surface area ($\mu A\ cm^{-2}$) by the potential difference (mV).

The cells were allowed to equilibrate for 20 min to establish a baseline short circuit current that did not vary by more than 0·4 $\mu A\ cm^{-2}$ in any 10 min interval thereafter, and ambroxol (10^{-6} M) was added to either the mucosal or submucosal solution. In control experiments, the cells were

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subjected to vehicle, the solvent of ambroxol (DMSO) alone. To assess cytotoxicity produced by ambroxol, after the short circuit current responses pleateaued, epithelial cells were subjected to treatment with isoprenaline (10^{-5} M) to increase the short circuit current (Smith et al 1982). In dose-response studies, ambroxol was cumulatively added to the mucosal or submucosal solution in molar increments.

To determine whether the ambroxol-induced changes in short circuit current were associated with Na^+ absorption and/or Cl^- secretion by epithelium, cells were pretreated for 30 min with the following drugs: diphenyl amine-2-carboxylate (10^{-4} M), a Cl^- channel blocker (DiStefano et al 1985); bumetanide (10^{-4} M), a Cl^- transport inhibitor (Widdicombe et al 1983); amiloride (10^{-4} M), a Na^+ channel blocker (Al-Bazzaz & Zevin 1984); ambroxol (10^{-6} M) was subsequently added.

Statistics

All values are expressed as means ± s.e. Statistical analysis was performed by one-way analysis of variance or the Newman-Keuls multiple comparison test, and a P value < 0.05 was considered significant.

Results

The effects of ambroxol (10^{-6} M) and its solvent DMSO (0·01%) alone on the bioelectric properties of canine cultured tracheal epithelium are given in Fig. 1 and Table 1. Addition of DMSO to the mucosal and submucosal solutions in the Ussing chamber had no effect on the short circuit current. Ambroxol did not alter the short circuit current when it was added to the mucosal side, but the submucosal addition of this drug decreased it from 8·6 ± 1·8 to 5·1 ± 0·9 μA cm^{-2} ($P < 0.01$, n = 10), accompanied by a corresponding decrease in potential difference and tissue conductance. In ambroxol-treated cells, subsequent administration of isoprenaline

Table 1. Effect of ambroxol on bioelectric properties of canine cultured tracheal epithelium.

	Short-circuit current (μA cm^{-2})	Transepithelial potential difference (mV)	Cell conductance (ms cm^{-2})
Baseline	8·6 ± 1·8	2·2 ± 0·3	3·9 ± 0·6
Ambroxol (10^{-6} M)			
Mucosal	8·2 ± 1·4	2·2 ± 0·4	3·7 ± 0·5
Submucosal	5·1 ± 0·9**	1·6 ± 0·2*	3·1 ± 0·4*

Values are means ± s.e.; n = 10. *$P < 0.05$, **$P < 0.01$, significantly different from baseline values.

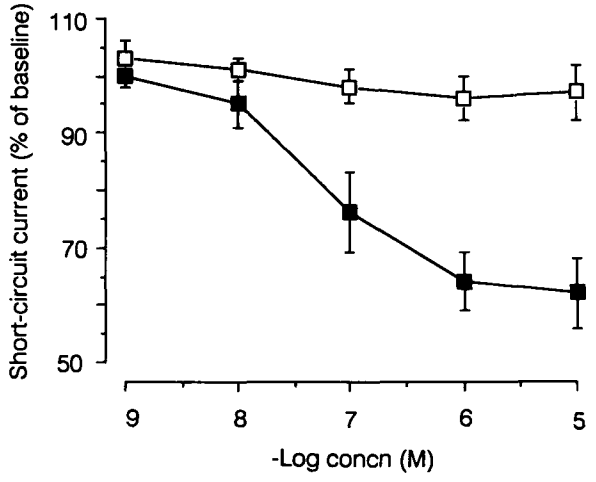

FIG. 2. Dose-dependent effect of ambroxol on short-circuit current of canine cultured tracheal epithelium. Ambroxol was cumulatively added to either the mucosal (□) or submucosal (■) solution. Responses are expressed as percent of baseline values obtained before the addition of the drug. Values are means ± s.e.; n = 9 for each group.

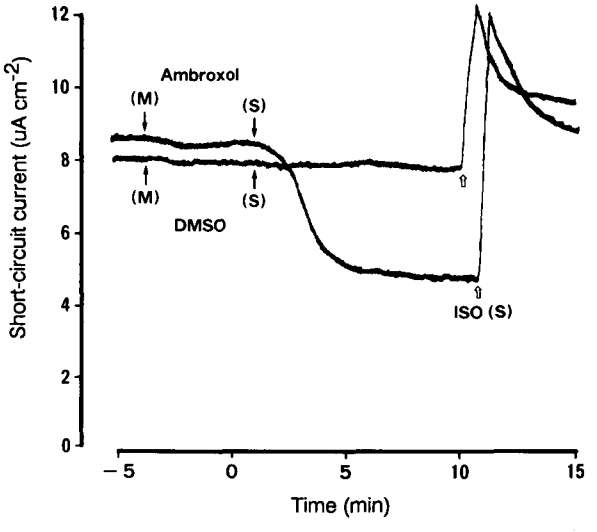

FIG. 1. A representative tracing showing the time course of the effects of ambroxol (10^{-6} M) or its solvent dimethylsulphoxide (DMSO, 0·01%) alone on short-circuit current of canine tracheal epithelium in culture. Each drug was added to the mucosal (M) or submucosal (S) solution in an Ussing chamber. After the short circuit current responses reached a plateau, isoprenaline (ISO, 10^{-5} M) was added to the submucosal solution.

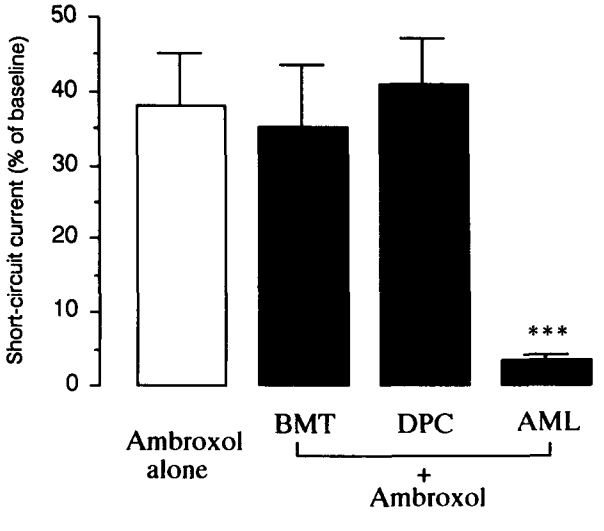

FIG. 3. Effects of bumetanide (BMT, 10^{-4} M), diphenylamine-2-carboxylate (DPC, 10^{-4} M) and amiloride (AML, 10^{-4}M) on the decrease in short-circuit current induced by submucosal addition of ambroxol (10^{-6} M) in canine cultured tracheal epithelium. Responses are expressed as means ± s.e.; n = 11 for each group. ***$P < 0.001$, significantly different from the response to ambroxol alone.

(10^{-5} M) to the submucosal solution increased the short circuit current to the same degree as in the control tissues, indicating that the decrease in short circuit current induced by ambroxol was not due to its cytotoxic actions.

Cumulative addition of ambroxol decreased the short circuit current in a dose-dependent manner; the maximal decrease from the baseline value and the concentration required to produce a half-maximal effect (IC50) was $39{\cdot}4 \pm 7{\cdot}2\%$ ($P < 0{\cdot}001$, $n = 9$) and 60 nM, respectively (Fig. 2). In contrast, mucosal ambroxol was without effect on the short circuit current at concentrations of up to 10^{-5} M.

Pretreatment of tracheal epithelium with bumetanide (10^{-4} M) or DPC (10^{-4} M) did not influence the inhibitory effect of ambroxol on the short circuit current, but amiloride (10^{-4} M) abolished the ambroxol (10^{-6} M)-induced decrease in short circuit current from $37{\cdot}9 \pm 7{\cdot}1$ to $3{\cdot}4 \pm 1{\cdot}2\%$ ($P < 0{\cdot}001$, $n = 11$) (Fig. 3).

Discussion

Our in-vitro studies demonstrate that ambroxol may selectively inhibit Na^+ absorption by canine tracheal epithelial cells. This is based on the findings that ambroxol elicited a dose-dependent decrease in short circuit current, and that this decrease was abolished in epithelial cells in which Na^+ absorption had already been blocked with amiloride (Al-Bazzaz & Zevin 1984) but not in epithelial cells pretreated with bumetanide, a Cl^- transport inhibitor (Widdicombe et al 1983), or diphenyl amine-2-carboxylate an inhibitor of Cl^- conductance at the apical membrane (DiStefano et al 1985). It is known that Na^+ absorption in airway epithelial cells is mediated not only by the amiloride-sensitive Na^+ channels located on the apical membrane but also by the Na^+-K^+-ATPase on the submucosal membrane. However, ambroxol cannot be inhibiting the submucosal Na^+-K^+-ATPase, since ambroxol-treated cells showed increases in short circuit current in response to the subsequent administration of isoprenaline to the submucosal solution similar to those of the control tissues. Furthermore, ambroxol cannot simply be acting on the apical Na^+ channels, as amiloride does, as ambroxol exerted its effect only when applied to the submucosal surface. Therefore, the action of ambroxol cannot easily be attributed to inhibition of either of the transport systems generally thought to be responsible for the Na^+ absorption. One possible mechanism could be that the binding sites for ambroxol are located on the submucosal membrane of epithelial cells and activation of these receptors may inhibit the apical Na^+ channels through intracellular signal transduction systems.

It has been recognized that tracheal epithelial cells secrete Cl^- and absorb Na^+ from the lumen (Boucher & Larsen 1988), and that the changes of electrochemical potential gradient due to these processes may cause the corresponding flow of water across the airway mucosa (Welsh et al 1980). Thus, it seems reasonable to speculate that the inhibition of epithelial Na^+ absorption by ambroxol may decrease the water absorption and, hence, increase the water composition in airway surface fluid, which may consequently decrease the viscosity of mucus. According to the theoretical model for mucus propulsion (Sleigh 1989), this process probably improves mucociliary transport functions in the respiratory tract. Thus ambroxol might be useful in patients with chronic airway diseases who have difficulty in sputum expectoration.

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