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Stimulation by menthol of Cl secretion via a Ca^{2+}-dependent mechanism in canine airway epithelium

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1 To investigate the effect of menthol on airway epithelial ion transport function, we studied the bioelectrical properties of canine cultured tracheal epithelium by Ussing's short-circuit technique *in vitro*.

2 Addition of menthol (10^{-3} M) to the mucosal but not the submucosal solution increased the short-circuit current (I_{sc}) from 6.2 ± 0.9 to 14.0 ± 2.2 μA cm^{-2} ($P < 0.001$), and this effect was accompanied by increases in transepithelial potential difference and conductance. The response was dose-dependent, with the maximal increase from the baseline value and the concentration required to produce a half-maximal effect (EC_{50}) being 6.4 ± 0.9 μA cm^{-2} ($P < 0.001$) and 40 μM, respectively.

3 Other cyclic alcohols, including menthone and cyclohexanol, had no effect on the electrical properties.

4 The menthol-induced increase in I_{sc} was not altered by pretreatment of the cells with amiloride, indomethacin, or propranolol but was abolished by diphenylamine-2-carboxylate, furosemide or substitution of Cl with iodide in the medium.

5 Menthol (10^{-3} M) increased cytosolic levels of free calcium ([Ca^{2+}]i) from 98 ± 12 to 340 ± 49 nM ($P < 0.01$) in fura-2-loaded tracheal epithelium but did not affect the intracellular adenosine 3',5'-cyclic monophosphate content.

6 These results suggest that menthol stimulates Cl secretion across airway epithelium, probably through a Ca^{2+}-dependent mechanism, and might thus influence mucociliary transport in the respiratory tract.

Keywords: Menthol; airway epithelium; ion transport; calcium; mucociliary transport

Introduction

Airway epithelial cells play an important role in the regulation of mucociliary transport, which seems to be dependent on ciliary beating, the rheological properties of airway surface fluid, and mucus production (Wanner, 1977). It is known that the amount and the rheological properties of airway surface fluid can be influenced by the ion transport function of the airway epithelium. In particular, the electrochemical potential gradient produced by secretion of Cl towards, and absorption of Na from, the airway lumen may promote the subsequent water secretion and absorption, respectively, across the airway mucosa (Welsh *et al.*, 1980).

Menthol, a cyclic alcohol which is appreciated because it produces a cooling sensation, has been widely used as a component of food and drink, tobacco, cosmetics, etc. It has been reported that menthol increases the sensitivity of cutaneous cold receptors by modulating Ca^{2+} currents of neuronal membranes (Schäfer *et al.*, 1986; Swandulla *et al.*, 1986), and that peppermint oil, of which menthol is a major constituent, improves the symptoms of irritable bowel syndrome through a direct action on intestinal smooth muscle (Rees *et al.*, 1979; Taylor *et al.*, 1984). Although there are many ways in which inhaled menthol vapour can contact the surface of the respiratory mucosa, its action on airway epithelial function is unknown. Therefore, to elucidate the effect of menthol on epithelial ion transport and its mechanism of action, we studied the biolectric properties of canine cultured tracheal epithelium under short-circuit conditions *in vitro*.

Methods

Preparation of epithelium

Mongrel dogs of either sex weighing 21 to 38 kg were anaesthetized with intravenous pentobarbitone sodium (35 mg kg^{-1}), and the trachea was removed. After dissection of submucosal tissue and blood vessels, the resected tissues were placed in fresh medium containing Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient F-12 (1:1) with 0.05% protease type XIV (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), and maintained at 4°C for 24 h. After mild agitation, the tissue sections were removed from the medium and cells were concentrated by centrifugation (1000 *g*, 10 min). The cell pellets were washed with medium containing 5% foetal calf serum to neutralize the protease. These cells were suspended in the mixture of DMEM and Ham's F-12 containing 5% foetal calf serum, 100 u ml^{-1} each of penicillin and streptomycin, 100 μg ml^{-1} gentamicin, 10 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 25 ng ml^{-1} epidermal growth factor, and 7.5 μg ml^{-1} endothelial cell growth supplement. Our preliminary studies showed that this preparation of cells was composed of 98–99% epithelial cells and 1–2% fibroblasts and other nonepithelial cells, and the viability was between 86 and 97%, as assessed by trypan blue exclusion. The cells were then seeded at a density of 1.5×10^6 cm^{-2} using 1 ml of medium per Linbro tissue culture multiwell plate (15 mm diameter, 18 mm deep, Flow Lab Inc., McLean, Virginia, U.S.A.), and grown on nuclepore polycarbonate filters (13 mm diameter, 0.45 μm pore size) at 37°C in a CO_2 incubator (95% air:5% CO_2). The medium was changed every 24 h and on the 10th day of incubation, the cells became confluent and were used for measurement of bioelectric properties. Observation of cultured cells by transmission electron microscopy revealed that typical tight junctions separated two distinct membranes: the membrane facing the overlying medium contained microvilli and glycocalyx, while the membrane facing the filter surface was relatively unspecialized. These cells were not ciliated and did not contain goblet cells but maintained the electrical properties resembling those of the native tissue (Al-Bazzaz & Cheng, 1979).

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Measurement of electrical properties

The short-circuit technique for measuring electrical properties of cultured epithelium has been described previously (Coleman *et al.*, 1984). Briefly, the filter on which tracheal epithelial cells were grown was mounted between Ussing chambers (0.5 cm^2 surface area) and bathed with Krebs-Henseleit solution of the following composition (in mM): Na 143.9, K 5.6, Ca 1.9, Mg 1.2, Cl 117.6, HCO_3 25.0, acetate 5.6, gluconate 3.8, H_2PO_4 1.3, SO_4 1.2 and glucose 5.6, oxygenated with 95% O_2:5% CO_2 and maintained at 37°C. Transepithelial potential difference (PD) was measured with two polyethylene bridges containing 3% agar in 1 M KCl, positioned within 1 mm of each side of the epithelial surface and connected to calomel electrodes (model 2080A-06T, Horiba Ltd, Tokyo, Japan) and a high-impedance voltmeter (model CEZ-9100, Nihon Kohden). Another pair of polyethylene bridges (3% agar in saline), positioned 10 mm from the orifice and connected to Ag/AgCl wires, was used to pass sufficient current through both the chamber and cells to bring the PD to zero. This short-circuit current (I_{sc}) was automatically corrected for solution resistance between the PD-detecting bridges, and recorded continuously on a pen recorder (model SR6335, Graphtec, Tokyo) except for 5 s every 3 min when the voltage clamp was turned off and the PD was recorded.

Tissue conductance (G) was calculated by dividing the measured I_{sc} per surface area by the PD. After a 20 min equilibration of cells when the PD did not vary by more than 0.2 $\mu A\ cm^{-2}$ in any 5-min intervals, cyclic alcohols including menthol, menthone and cyclohexanol (10^{-3} M) were added to either the mucosal or submucosal solution in the chamber. To assess the concentration-dependent relationship between menthol and I_{sc}, menthol was cumulatively added from 10^{-6} to 10^{-3} M in half-molar increments, during which the highest I_{sc} value recorded at each concentration was determined.

To assess whether the responses of I_{sc} were derived from Na absorption and/or Cl secretion by the epithelium, cells were pretreated for 30 min with each of the following: mucosal amiloride (10^{-4} M), a Na channel blocker (Al-Bazzaz & Zevin, 1984); mucosal diphenylamine-2-carboxylate (10^{-4} M), a Cl channel blocker (DiStefano *et al.*, 1986); submucosal furosemide (10^{-4} M), an inhibitor of the Na/K/2Cl cotransporter (Widdicombe *et al.*, 1983); or Cl-free medium on both sides in which Cl was substituted with iodide, which cannot be transported across the canine tracheal epithelium (Widdicombe & Welsh, 1980). Menthol (10^{-3} M) was then added to the chamber and the I_{sc} responses were compared with those of cells without pretreatment. To test possible involvement of prostaglandin synthesis or β-adrenoceptor activation in the action of menthol, we also examined the effects of pretreatment of cells with indomethacin (10^{-5} M) and propranolol (10^{-5} M).

Measurement of intracellular adenosine 3',5'-cyclic monophosphate (cyclic AMP)

We measured intracellular cyclic AMP levels, one of the important determinants for ion transport function (Welsh, 1987). The tracheal epithelial cells were incubated in the chambers, and menthol, menthone and cyclohexanol (10^{-3} M) were each added to the mucosal solution. For a positive control experiment, isoprenaline (10^{-6} M) was added to the submucosal solution. After 20 min, epithelia were removed from the chambers, and sonicated in a bath-type sonicator in ice-cold 10% trichloroacetic acid. After the extraction of trichloroacetic acid with ether, the residue was dissolved in acetate buffer. The cyclic AMP levels were determined in duplicate by a radioimmunoassay method (Brooker *et al.*, 1979), normalized for the protein content of the cells as determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, and corrected for the percentage recovery of [^{125}I]-cyclic AMP added as a tracer.

Measurement of $[Ca^{2+}]i$

Canine tracheal epithelial cells were cultured on round coverslips (15 mm diameter, Matsunami Ltd., Tokyo) coated with human placental collagen (20 $\mu g\ cm^{-2}$). After confluence was achieved, the coverslip was washed with Hank's balanced salt solution containing 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) at pH 7.4 and loaded with 2×10^{-6} M acetoxymethyl ester of fura-2 (fura 2-AM, Dojin Lab, Kumamoto, Japan) for 20 min at 37°C. After washing, the coverslip was held with a rigid holder in a continuously stirred cuvette containing HEPES-buffered Hank's solution maintained at 37°C, and the fluorescence intensity was measured by a spectrophotometer (model CAF-100, Japan Spectroscopic Co., Tokyo). Excitation at 340 and 380 nm was automatically exchanged at a rate of 50 Hz, and the emitted light from cells was detected by a photomultiplier tube through a 500 nm filter.

Maximal (R_{max}) and minimal (R_{min}) values for the ratio were determined in the presence of ionomycin (10^{-5} M) and ethylene glycol *bis* (β-amino-ethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA, 5×10^{-3} M), and $[Ca^{2+}]_i$ values were calculated by the formula described by Grynkiewicz *et al.* (1985).

Drugs

The following drugs were used: menthol (2-isopropyl-5-cyclohexanol), amiloride, furosemide, indomethacin, (±)-propranolol, ionomycin, EGTA (Sigma Chemical Co.), diphenylamine-2-carboxylate, menthone, and cyclohexanol (Nacalai Tesque, Kyoto, Japan). Menthol was dissolved in dimethylsulphoxide (DMSO) and subsequently diluted in Krebs-Henseleit solution. The highest final concentration of DMSO in the Ussing chamber was 0.1%. Menthone and cyclohexanol were diluted in Krebs-Henseleit solution. Our preliminary studies showed that the vehicle (diluted DMSO) had no effect on the electrical properties of canine cultured tracheal epithelium.

Statistics

All values are expressed as means ± s.e.mean. Statistics analysis was performed by ANOVA or Newman-Keul multiple comparison test, and a P value of less than 0.05 was considered significant.

Results

Electrical properties

Addition of menthol (10^{-3} M) to the mucosal solution elicited an increase in I_{sc} of canine cultured tracheal epithelium, which peaked within 10 min (6.2 ± 0.9 to 14.0 ± 2.2 $\mu A\ cm^{-2}$, $P < 0.001$, $n = 9$) and remained elevated during the 30 min observation period (Figure 1). This menthol-induced increase in I_{sc} was accompanied by corresponding increases in PD and G ($P < 0.01$ and $P < 0.05$, respectively, $n = 9$) (Table 1). In contrast, submucosal addition of menthol did not alter these electrical properties. Menthone and cyclohexanol (10^{-3} M) when added to either the mucosal or submucosal solution had no effect on I_{sc} (Figure 1).

Cumulative addition of menthol to the mucosal solution increased I_{sc} in a concentration-dependent manner (Figure 2). The maximal increase from the baseline value (ΔI_{scmax}) and the concentration required to produce a half-maximal effect (EC_{50}) were 6.4 ± 0.9 $\mu A\ cm^{-2}$ ($P < 0.001$, $n = 10$) and 40 μM, respectively. The change of I_{sc} in response to the submucosal menthol did not reach a significant level.

Incubation of cells with amiloride, diphenylamine-2-carboxylate, furosemide and Cl-free medium *per se* decreased

Table 1 Effect of menthol on bioelectric properties of canine cultured tracheal epithelium

	Mucosal			*Submucosal*		
	Before	*After*	*Significance*	*Before*	*After*	*Significance*
I_{sc} ($\mu A\ cm^{-2}$)	6.2 ± 0.9	14.0 ± 2.2	$P<0.001$	6.8 ± 0.7	7.3 ± 0.7	NS
PD (mV)	2.2 ± 0.4	3.9 ± 0.4	$P<0.01$	2.3 ± 0.3	2.7 ± 0.4	NS
G ($mS\ cm^{-2}$)	2.8 ± 0.3	3.6 ± 0.4	$P<0.05$	2.9 ± 0.4	2.3 ± 0.3	NS

Definitions of abbreviations: I_{sc}, short-circuit current; PD, transepithelial potential difference; G, conductance; NS, not significant. Menthol (10^{-3} M) was added to either the mucosal or the submucosal solution of the Ussing chamber. Values are means ± s.e.; $n = 9$.

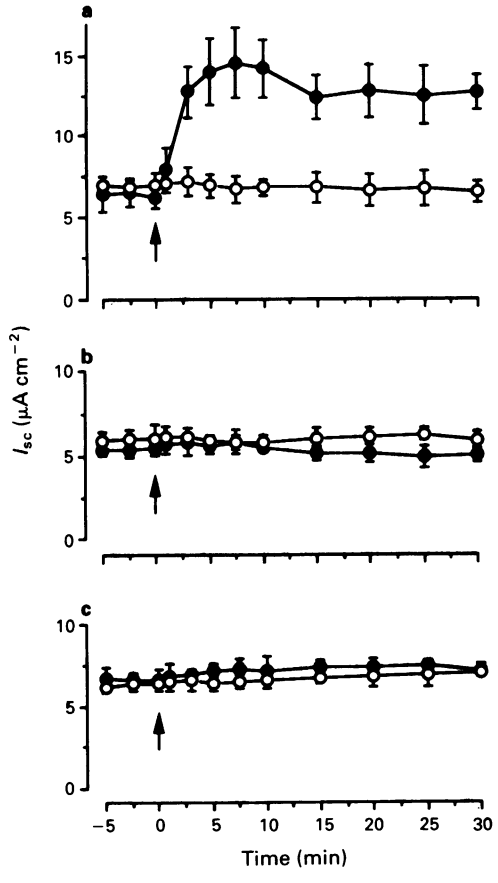

Figure 1 Time-course of the effects of menthol (10^{-3} M, a), menthone (10^{-3} M, b), and cyclohexanol (10^{-3} M, c) on short-circuit current (I_{sc}) of canine cultured tracheal epithelial cells. After establishing the baseline I_{sc}, each drug was added at time 0 (arrow) to either the mucosal (●) or submucosal solution (○) of the Ussing chamber. Each point represents the mean (with s.e.mean) of 9 experiments.

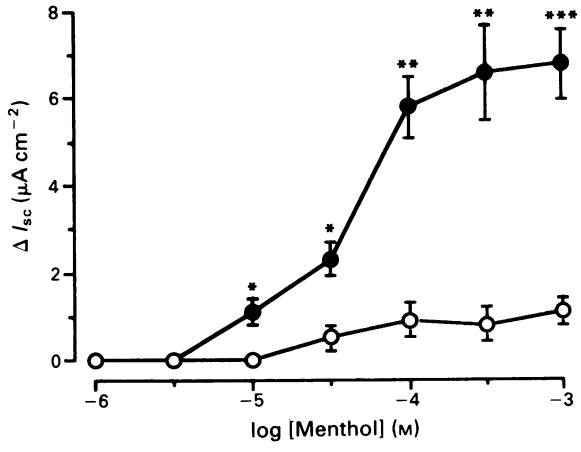

Figure 2 Concentration-dependent effect of menthol on short-circuit current (I_{sc}) of canine cultured tracheal epithelium. Menthol was added to either the mucosal (●) or submucosal (○) solution. Responses are expressed as the increase in I_{sc} (ΔIsc) from the baseline values obtained before the addition of menthol. Each point represents the mean (with s.e.mean) of 10 experiments. $*P<0.05$; $**P<0.01$; $***P<0.001$, significantly different from the baseline I_{sc}.

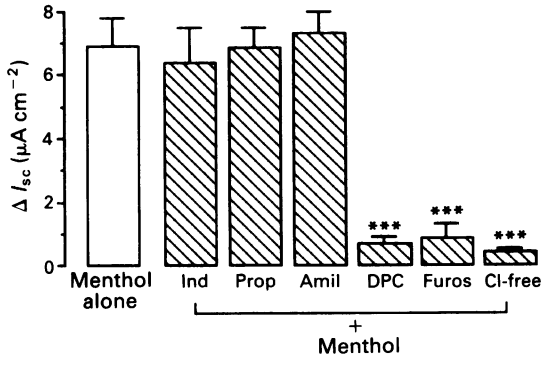

Figure 3 Effects of indomethacin (Ind, 10^{-5} M), propranolol (Prop, 10^{-5} M), amiloride (Amil, 10^{-4} M), diphenylamine-2-carboxylate (DPC, 10^{-4} M), furosemide (Furos, 10^{-4} M), and substitution of Cl in the bathing medium with iodide (Cl-free) on the increase in short-circuit current (ΔIsc) induced by mucosal addition of menthol (10^{-3} M) in canine cultured tracheal epithelial cells. Data are means (with s.e.mean) of 7 experiments. $***P<0.001$, significantly different from the response to menthol alone.

baseline I_{sc} of 3.5–9.4 $\mu A\ cm^{-2}$ by 1.9 ± 0.3, 4.2 ± 0.5, 3.7 ± 0.4 and 5.5 ± 0.4 $\mu A\ cm^{-2}$, respectively ($n = 7$, in each case). The increase in I_{sc} induced by the subsequent addition of menthol (10^{-3} M) to the mucosal solution was not altered by amiloride but greatly attenuated by diphenylamine-2-carboxylate, furosemide, and Cl-free medium ($P<0.001$, $n = 7$) (Figure 3). Pretreatment of cells with indomethacin and propranolol had no effect on the I_{sc} response to menthol.

Intracellular cyclic AMP levels

Incubation of epithelial cells with menthol (10^{-3} M), menthone (10^{-3} M), or cyclohexanol (10^{-3} M) did not significantly alter intracellular cyclic AMP levels (Figure 4). As a positive control experiment, the β-adrenoceptor agonist isoprenaline (10^{-6} M) increased cyclic AMP levels from 3.6 ± 0.29 to 121.5 ± 16.0 pmol mg^{-1} protein ($P<0.01$, $n = 5$).

Intracellular Ca^{2+} concentration

As shown in Figure 5, addition of menthol (10^{-3} M) increased $[Ca^{2+}]_i$ in canine cultured tracheal epithelium from 98 ± 12 to 340 ± 49 nM ($P<0.01$, $n = 5$), whereas menthone (10^{-3} M) and cyclohexanol (10^{-3} M) had little effect. The menthol-induced $[Ca^{2+}]_i$ response reached a plateau within 3 min and remained elevated as long as the drug was present.

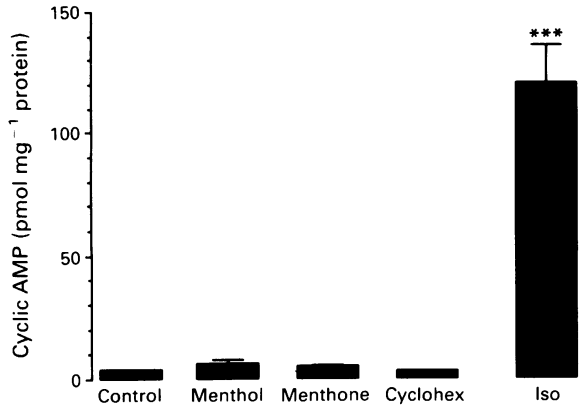

Figure 4 Effects of menthol, menthone and cyclohexanol (Cyclohex) at 10^{-3} M on cyclic AMP levels in canine tracheal epithelium. As a positive control, the effect of isoprenaline (Iso, 10^{-6} M) on cyclic AMP levels was assessed. Data are means (with s.e.mean) of 5 experiments. $***P<0.001$, significantly different from control tissues.

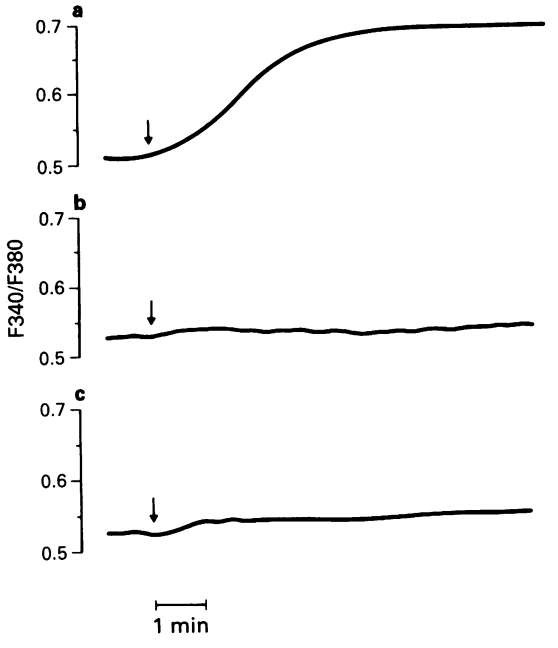

Figure 5 Typical recordings of fura-2 fluorescence ratios in canine tracheal epithelial cells exposed to menthol (a), menthone (b) and cyclohexanol (c) at 10^{-3} M, taken from 9–20 experiments. Immediately after the addition (arrows) of the compounds, menthol increased $[Ca^{2+}]_i$ whereas menthone and cyclohexanol had no effect.

Discussion

Our *in vitro* studies demonstrate that menthol alters the bioelectric properties of canine cultured tracheal epithelial cells. It is known that the alteration in the electrical properties of the airway epithelium is closely associated with functional changes of electrolyte transport across the cell consisting of Na absorption and Cl secretion (Boucher & Larsen, 1988). Although we did not measure ion fluxes directly in the present study, our results indicate that menthol may selectively stimulate the secretion of Cl across tracheal epithelial cells from the submucosal side toward the airway lumen. This conclusion is based on the following findings. First, menthol produced a concentration-dependent increase in I_{sc}, a bioelectric parameter that reflects net movement of actively transported ions (MacKnight *et al.*, 1980), and which was accompanied by increases in PD and G. Second, this increase in I_{sc} was not influenced by pretreatment of cells with amiloride, a Na channel blocker (Al-Bazzaz & Zevin, 1984), but was abolished by the Cl channel blocker, diphenylamine-2-carboxylate (DiStefano *et al.*, 1986), the inhibitor of the Na/K/2Cl cotransporter furosemide (Widdiscombe *et al.*, 1983), and the substitution of Cl in the medium with iodide, an anion that cannot be transported across the airway epithelium (Widdicombe & Welsh, 1980).

Because menthol is an alcohol, it could have acted on epithelial cells by inducing membrane labilization and/or modifying membrane fluidity due to its lipophilic properties (Grisham & Barnett, 1973; Haydon *et al.*, 1977). However, neither cyclohexanol, a highly lipid soluble cyclic alcohol from which menthol is derived, nor menthone, in which the hydroxy group of menthol is replaced with a keto group, produced any alteration in bioelectric properties, suggesting that the stimulation of Cl secretion may be specific for menthol. This specificity has also been reported in dihydropyridine-insensitive Ca^{2+} channels in human neuroblastoma cells (Sidell *et al.*, 1990).

In contrast to the responses to the mucosal addition of menthol, this drug did not alter the electrical properties of tracheal epithelium when it was added to the submucosal side. The reason for this difference is uncertain, but one possibility is that specific receptors for menthol could be located on the mucosal membrane rather than on the submucosal membrane of the epithelium.

It has been shown that activation of β-adrenoceptors can stimulate Cl secretion (Welsh, 1987) and that several agents stimulate Cl secretion through the synthesis and the release of cyclo-oxygenase products of arachidonic acid metabolism in canine tracheal mucosa (Eling *et al.*, 1986). To test the possible involvement of these mechanisms in the action of menthol, we examined the effects of the blockade of β-adrenoceptors and the cyclo-oxygenase pathway with propranolol and indomethacin, respectively. We found that the menthol-induced increase in I_{sc} was not reduced by these pharmacological blocking agents. Thus, the menthol-induced Cl secretion does not appear to be mediated by β-adrenoceptors or cyclo-oxygenase products.

Intracellular cyclic AMP and cytosolic Ca^{2+} are important second messengers regulating Cl secretion in the airway epithelium (Welsh, 1987). In the present study, cyclic AMP levels in tracheal epithelial cells were not changed by 10^{-3} M menthol, a concentration sufficient to increase epithelial I_{sc}. In contrast, the same concentration of menthol substantially increased $[Ca^{2+}]_i$ indicating that the effect of menthol on electrical properties may be associated with an increase in cytosolic free Ca^{2+}. In most cell types, a rapid transient increase and a sustained elevation of $[Ca^{2+}]_i$ may be derived from the Ca^{2+} release from intracellular stores and the Ca^{2+} influx from the extracellular solution, respectively (Williamson & Monck, 1989). Because the response of $[Ca^{2+}]_i$ to menthol was gradual and long-lasting, we speculate that it might be dependent on influx from the extracellular solution. To confirm this, further studies on $[Ca^{2+}]_i$ kinetics will be required.

In contrast to our findings, it has been reported that menthol does not stimulate but, instead, potently blocks Ca^{2+} currents through voltage-activated channels in cultured dorsal root ganglion cells from chick and rat embryos (Swandulla *et al.*, 1987), and that peppermint oil, of which menthol is a major constituent, improves some symptoms of irritable bowel syndrome (Swandulla *et al.*, 1986; Taylor *et al.*, 1984) probably through a mechanism involving Ca^{2+} antagonism (Hills & Aaronson, 1991). The reason for this contradictory action of menthol is uncertain, but it could be due to the difference in the cell types.

Active transport of Cl across the tracheal epithelium correlates to the movement of water toward the lumen (Welsh *et al.*, 1980). This water movement probably affects the depth of the periciliary sol layer of airway surface fluid and hydrates

airway mucus that interacts with ciliary function (Nadel *et al.*, 1985). Finally, it is appreciated that high concentrations of menthol are required to produce our observed effects but comparable concentrations are necessary in other systems (Swandulla *et al.*, 1986; Siddell *et al.*, 1990). Moreover, the concentration of menthol that may be achieved in the airways is unknown and it thus remains uncertain whether the drug may affect mucociliary transport function *in vivo*.

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