Mucociliary clearance and submucosal gland secretion in the ex vivo ferret trachea

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Jeong JH, Joo NS, Hwang PH, Wine JJ. Mucociliary clearance and submucosal gland secretion in the ex vivo ferret trachea. Am J Physiol Lung Cell Mol Physiol 307: L83–L93, 2014. First published May 2, 2014; doi:10.1152/ajplung.00009.2014.—In many species submucosal glands are an important source of tracheal mucus, but the extent to which mucociliary clearance (MCC) depends on gland secretion is unknown. To explore this relationship, we measured basal and agonist-stimulated MCC velocities in ex vivo tracheas from adult ferrets and compared the velocities with previously measured rates of ferret glandular mucus secretion (Cho HJ, Joo NS, Wine JJ. Am J Physiol Lung Cell Mol Physiol 299: L124–L136, 2010). Stimulated MCC velocities (mm/min, means ± SE for 10- to 35-min period poststimulation) were as follows: 1 μM carbachol: 19.1 ± 3.3 > 10 μM phenylephrine: 15.3 ± 2.4 = 10 μM isoproterenol: 15.0 ± 1.9 = 10 μM forskolin: 14.6 ± 3.1 > 1 μM vasoactive intestinal peptide (VIP): 10.2 ± 2.2 >> basal (t15): 1.8 ± 0.3; n = 5–10 for each condition. Synergistic stimulation of MCC was observed when low concentrations of carbachol (100 nM) and isoproterenol (300 nM). Bumetanide inhibited carbachol-stimulated MCC by ~70% and abolished the increase in MCC stimulated by forskolin + VIP, whereas HCO3−-free solutions did not significantly inhibit MCC to either intracellular Ca2+ concentration or intracellular cAMP concentration ([cAMP]i)-elevating agonists. Stimulation and inhibition of MCC and gland secretion differed in several respects: most importantly, elevating [cAMP]i increased MCC much more effectively than expected from its effects on gland secretion, and bumetanide almost completely inhibited [cAMP]i-stimulated MCC while it had a smaller effect on gland secretion. We conclude that changes in glandular fluid secretion are complexly related to MCC and discuss possible reasons for this.

Cystic fibrosis (CF), which is caused by defects in the anion channel CFTR, is characterized by viscous mucus in all mucus-producing organs (17, 44). Because CFTR functions primarily as an anion channel, defective mucus is thought to result from defective secretion of anions and anion-mediated fluid secretion, with the precise roles of pH, HCO3−, chelation of Ca2+, and water still under investigation (44). In the airways, loss of CFTR impairs the function of both surface epithelia and glands (13, 26–30, 60), without inherent effects on ciliary beat frequency (CBF) (47). Exactly how these CFTR-dependent defects alter mucociliary transport is of great interest, because of their potential bearing on CF lung disease.

Submucosal glands are an important source of tracheal mucus in species such as humans, pigs, and ferrets, and gland-free tracheas are more prone to infections than gland replete tracheas (16). MUC5B is the predominant mucin released from awake submucosal glands (5, 57). In an important article, MUC5B, but not MUC5AC, was shown to be required for airway defense in the mouse (46). In the mouse, most of this critical mucin is released continuously from Clara cells (75), but at least in tracheas of larger animals, submucosal glands are likely to be the source, since tracheal Clara cells (or at least the Clara cell marker CCSP) are scant (15). Therefore, one hypothesis is that diminished and/or altered mucin secretion from CF airway glands (6–8, 13, 14, 27, 29, 60) contributes to defective clearance and a reduced ability to resist infections (70), with infections causing a further decrease in the transportability of mucus (56).

The ferret trachea is a good model system for studying gland secretion and mucociliary transport. Ferrets are born with an immature tracheal epithelium that lacks glands, is sparsely ciliated (37), has relatively few cells expressing high levels of CFTR (54), and also lacks MCC (64). Cilia, glands, and CFTR expression (54) develop progressively over the first 4 wk (37, 54) and so does MCC, which at week 4 reaches adult values of 11 mm/min (measured in vivo in anesthetized ferrets) (64).

CF ferrets develop human-like lung disease (59, 60). This makes them potential models for investigating the relationship between gland secretion, mucociliary transport, and CFTR function. In particular, a recent comparison of tracheal MCC in wild-type and CF (CFTR−/−) ferrets showed an approximately sevenfold decrease in the latter (59). To help determine the role of submucosal glands in MCC, a previous parametric study of ferret tracheal gland secretion measured rates of mucin secretion from individual glands in response to agonists and transport inhibitors (12). With those data in hand, the present study was undertaken to compare MCC velocities to the same array of agonists and anion transport inhibitors.

MUCUS CLEARANCE IS AN ESSENTIAL innate defense mechanism for mammalian airways that depends on mucociliary transport [mucociliary clearance (MCC)] and cough clearance (33). When mucociliary clearance is impaired, the airways are prone to infection and obstruction, which are mutually exacerbating. Impairments can be caused by ciliary dysfunction or by alterations in the production or properties of mucus and can be initiated by genetic (e.g., primary ciliary dyskinesia and cystic fibrosis) or environmental (e.g., smoking) insults. Once impaired for any reason, infection follows and causes further impairment, which increases reliance on cough clearance and the recruitment of the adaptive immune system to help resolve the infection and clear the airways. If these prove inadequate, chronic disease results, bronchiectasis develops, and lung function is progressively lost.

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The analysis of ion transport inhibitors like bumetanide, dimethylamiloride, and 5-nitro-2-(3-phenylpropylamino)benzoic acid is useful because agonists stimulate both CBF and anion-mediated fluid secretion, but these ion transport inhibitors did not affect CBF (8). Also, the loss of CFTR affects both Cl⁻ and HCO₃⁻ secretion, and inhibitors can help sort out the contributions of each anion. In ex vivo pig tracheas with ACh as the agonist, blocking both Cl⁻ and HCO₃⁻ secretion almost eliminated MCC (8). To look for differential effects, Ballard and colleagues (14) reduced Cl⁻ and HCO₃⁻ secretion independently. Of great interest, they found opposite effects on basal and ACh-stimulated MCC velocity. Bumetanide reduced MCC velocity to near zero, but removal of HCO₃⁻ eliminated MCC (8). To look for differential effects, Ballard et al. (14) reduced Cl⁻ by 10–20% with 5-nitro-2-(3-phenylpropylamino)benzoic acid. This reduced MCC by 25%, whereas indomethacin and HCO₃⁻-free solutions reduced MCC by 50% (14). Those findings were the point of departure for the present studies.

**MATERIALS AND METHODS**

**Tissues and experimental setup.** The experimental procedures were approved by the Stanford University Institutional Animal Care and Use Committee. Ferret tracheas (Mustela putorius furo, n = 89) were obtained ~1 h postmortem (pentobarbital sodium injection) from 2-mo-old to 6-mo-old female ferrets that had been used in other experiments in which they served as controls. Tissues (usually 6 tracheas) were transported to the laboratory in cold Physiosol solution (Abbott Laboratories) and kept at 5°C until use, usually within 6 h. The trachea was obtained from just below the larynx to just above the carina. The dorsal muscular portion of the trachea was cut along its entire length, being careful to avoid touching the ventral and lateral mucosa. The opened trachea with cartilage intact was pinned mucosal-side-up in a trough made of Silgard (Dow Corning) with the cut edges separated to allow visualization of the tracheal mucosa; during this procedure the trachea was submerged in Krebs-Bicarbonate buffer that warmed from ~5°C at the start of the procedure to near-room temperature at the end. Before placement in the observation chamber, the trough with the pinned-out trachea was flicked with a sharp wrist movement five times to remove excess buffer from the tracheal surface. This procedure was arrived at empirically. If excess buffer remained on the mucosa, the ink particles swirled in eddies, whereas no movement was observed if the surface became too dry.

Once in the observation chamber (Fig. 1, A and B), the trough holding the trachea was filled with ~2 ml of bath solution so that the serosa of the trachea was submerged in the bath solution, and a plastic cover with a clear glass window was tightly fitted to the chamber. The tissue was continuously exposed to warmed, humidified gas, and the tissue was warmed from room temperature to 37°C at a rate of 0.9 mm/min at a temperature-controlled system (Standard Heatblock; VWR Scientific). The atmosphere of the chamber was 95% O₂-5% CO₂ gas except for HCO₃⁻-free experiments, when it was 100% O₂. Submicroliter droplets of fluid placed in the chamber to test its humidity shrank during the experiment, indicating the high humidity was insufficient to eliminate evaporative loss. Tissues were bathed in Krebs-Ringer bicarbonate buffer consisting of the following (in mm): NaCl 115, K₂HPo₄ 2.4, KH₂PO₄ 0.4, NaHCO₃ 25, CaCl₂, 1.2 MgCl₂, 1.2 KCl, and 10 glucose. Indomethacin (1 μM) was added to reduce tissue exposure to endogenously released prostaglandins. Osmolarity was adjusted to 290 ± 5 mosM with a vapor pressure osmometer (model 5500; Wescor), and pH was verified to be 7.4 (Orion 420A pH meter) after the solution was bubbled with 95% O₂-5% CO₂. When warmed to 37°C, the pH increased to 7.65–7.7. We did not compensate for this change, but in four tracheas we compared carbachol-stimulated MCC at both pH values by starting with one bath pH and then changing to the other in a balanced order. The transport velocities in mm/min were 20 ± 2.1 at pH 7.7 and 22 ± 3.8 at pH 7.4 (NS). For HCO₃⁻-free experiments, the 25 mM HCO₃⁻ in the Krebs solution were replaced with 25 mM HEPES that had been pregressed with 100% O₂. The pH for the HEPES solution was 7.4 after it was gassed with 100% O₂.

**Fig. 1.** Overview of the experimental setup used to measure mucociliary clearance (MCC) in 2 ferret tracheas. A: sealed chamber for measuring MCC. Temperature and humidity were controlled, and once sealed, the chamber was not reopened. Solution changes and ink particle additions were made via small ports that were kept closed except when pipet tips were introduced. B: 2 tracheas were mounted side by side in the chamber with bath compositions that could be separately controlled. Ink particles were deposited in region a shown by the orange rectangle and were followed through the region indicated by the arrows. Particles and mucus accumulated at the proximal (rostral) end and were periodically removed. Small scale divisions are 1 mm. C: Xerox ink particles used to track MCC. D: decline in MCC rate during first 15 min in chamber as Krebs solution is transported from the surface. For particles deposited in region a that traversed the region indicated by solid area (filled points and solid line), the mean basal MCC velocity was 7.1 ± 0.9 mm/min at t₀ and dropped to 1.8 ± 0.3 mm/min at t₁₅, (n = 27–35, P < 0.001 vs. t₀ vs. t₁₅). When particles were placed at point b and traversed the region indicated by dashed arrow (open points and dashed line), the mean basal MCC velocity was 1.4 ± 0.2 mm/min at t₀ and dropped to 0.2 ± 0.1 mm/min at t₁₅ (n = 27, P < 0.001 for t₀ vs. t₁₅).
Measurement of MCC velocity. Each full-length trachea was gently stretched and pinned with nine pins per side, placed at the most extreme ends and then approximately equidistant. To measure MCC, we periodically applied X-ray film powder (particle size 10 μm; Fig. 1C) to the mucosal surface at the distal (caudal) end of trachea between pins 2 and 3. The toner was held in a modified pipet tip that fit snugly into a small port in the side of the chamber; a narrow plume of particles was applied by gently tapping the pipet tip. This method avoided alteration of the humidified atmosphere during application. Digital images were captured automatically at 20-s intervals using a 2-megapixel digital microscope (ZipScope; Aven, Ann Arbor, MI) and its software. Stored images were analyzed using National Institutes of Health ImageJ software (http://rsb.info.nih.gov/ij/). After the measurement scale was set, the fastest moving cluster of particles was measured for three consecutive 20-s periods and the average of the measurements was taken as the peak transport velocity for that time; this procedure was repeated every 5 min throughout the 45-min recording period. To represent stimulated MCC velocity with a single number, we averaged each of the 5-min measurement periods for times 25–45 (starting ~10 min after agonist addition).

MCC transport velocities are highly heterogeneous, which requires a set of rules to determine data exclusion. Some heterogeneity is intrinsic to the system, and some is introduced by procedures needed for the ex vivo experiments. We attempted to minimize the contributions of three artifacts. 1) Faster movement of particles was often seen near the lateral margins where pins were placed and some wicking of bath fluid occurred. 2) The transported fluid pooled at the proximal (rostral) end of the trachea, and MCC slowed as particles reached this area. 3) The distal end of the trachea, which would normally be receiving airway surface liquid (ASL) transported from the more distal Airways, receives nothing in this isolated tracheal preparation, and the amount of fluid generated by the trachea in basal and some stimulated conditions is not sufficient to overcome absorption and transport of ASL from this region, causing a gradual drying leading to slowing and then cessation of MCC that progresses in a proximal-distal direction. All of these artifacts were excluded from our measurements, which were restricted to the earliest and fastest moving particles along track a in Fig. 1B. Because of these restrictions, we needed to use full-length tracheas from adult ferrets.

Stimulation and inhibition protocols. After placement in the chamber and a 15-min warming period, we began the observation period. Each plotted point represents the mean of three 20-s measurement periods. Usually the preparation reached 37°C a few minutes before we applied the first particles and began imaging. We observed the basal velocity for 15 min and then replaced the warmed bath with an equal volume of 37°C bath containing agonists or inhibitors. We changed the bath by drawing it out with a syringe through tubing tightly fitted to a port in the side of the chamber; a second syringe introduced the fresh, warmed bath + agonists. The port was plugged when not in use. Sometimes we tilted the chamber slightly to collect all of the bath fluid. After agonists were introduced, MCC was monitored for 30 min. Additional particles were added as required.

To assess the effects of anion-mediated secretion on ferret MCC, we inhibited Cl \(^{-}\) secretion with 100 μM bumetanide to block the Na\(^{+}\)-K\(^{-}\)-2 Cl\(^{-}\) cotransporter. We inhibited HCO\(_3\) \(^{-}\) secretion by replacing it with 25 mM HEPES and gassing with 100% O\(_2\), and we combined the treatments to eliminate both anions. We added the inhibitory bath as soon as the trachea was placed into the chamber to begin warming and included them with the agonist baths so that they were continuously present during the experiment. We compared anion inhibitory effect on two agonists: 1 μM carbachol to elevate intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and 1 μM vasoactive intestinal peptide (VIP) + 10 μM forskolin to elevate intracellular cAMP concentration ([cAMP]). The VIP/forskolin combination was chosen to match the formulation used to study inhibition of gland mucus secretion (12).

Reagents. We purchased all chemicals from Sigma. We dissolved carbachol (cholinergic agonist), phenylephrine (α\(_1\)-adrenergic agonist), isoproterenol (β\(_2\)-adrenergic agonist), and VIP in deionized distilled water; forskolin in DMSO; and bumetanide in 0.5 N NaOH solution. All drugs were made as stock solutions with concentrations 1,000 times the expected final concentration, and 20-μl aliquots were stored at ~20°C until the day of use when they were diluted 1:1,000 with the appropriate bath solution.

Statistics. We used SPSS (Version 21 for Windows) or Excel for statistical analysis. Values are means ± SE unless otherwise indicated. For the stimulation data (see Figs. 2 and 4), we used one way ANOVA to compare the velocities at t\(_{15}\) and t\(_{25–45}\), omitting the transitional t\(_{20}\) velocity. The data for the MCC velocities (see Fig. 8) for the last three time points were averaged for each animal and those data were used to compute the group means; Kruskal-Wallis one-way ANOVA was used to evaluate differences among group means; and post hoc comparisons were with Mann-Whitney U-test. Differences were considered significance if P < 0.05.

Supplemental material. For examples of experiments see accompanying .avi files. (Supplemental Material for this article is available online at the Journal website.)

RESULTS

Basal MCC velocities declined quickly and were slower in distal regions. In this experimental model, the isolated, unstimulated trachea does not replace ASL as fast as it is depleted by MCC and absorption. This occurs in part because the trachea has been deprived of its central innervation and its blood supply and because isolation deprives the trachea of the ASL that is normally generated by the extensive epithelial surface area of the distal lung and transported into the trachea. Accordingly, ASL depletion and slowing of MCC progressed from the distal to the proximal trachea.

We documented this by measuring “basal” MCC in full-length tracheas from 27 to 35 ferrets for a 15-min period that started shortly after the preparation reached 37°C. We measured MCC in two portions of the trachea (Fig. 1B, tracks a and b). The MCC velocity of track a was initially five times higher than track b and declined over time in both regions, quickly reaching stasis in the region covered by track b (Fig. 1D).

These initial velocities primarily reflect the extent of residual fluid (Krebs) on the mucosal surface when the tracheas are mounted, because they could be increased or decreased by the extent to which we removed excess fluid before mounting (see MATERIALS AND METHODS). After most of this fluid has been transported to the proximal trachea, transport velocities reached 1.8 ± 0.3 mm/min along track a. This was the starting point for the stimulation experiments, which only measured MCC in the region of the trachea shown by track a in Fig. 1B.

Carbachol and phenylephrine stimulated increased MCC velocities. Parasympathetic cholinergic input is a potent and efficacious stimulus for mucus secretion from single submucosal glands in every species where it has been tested (12, 13, 22, 31, 32, 70). In ex vivo ferret tracheas, the cholinergic agonist carbachol was the most potent agonist for mucus secretion from submucosal glands among the five that were tested: carbachol, phenylephrine, isoproterenol, forskolin, and VIP (12). To evaluate its effect on MCC in this system, we applied 1 μM carbachol within 3 min following the end of the 15-min basal period, which caused a rapid increase in MCC that was sustained for as long as we followed it (Fig. 2A). The average MCC velocity just before carbachol addition (t\(_{15}\) was
A limited set of dose-response experiments was carried out for carbachol using concentrations from 0.1 to 10 M (Fig. 3, A and B). Under the conditions of our experiments, 0.1 M was subthreshold and 1 M was maximal. Considering the limited number of experiments at each concentration other than 1 M, (0.1, 3, and 10 M were each tested only in a single trachea), the dose-response relation was in reasonable agreement with gland secretion rates obtained previously (12) (Fig. 3B).

Forskolin, VIP, and isoproterenol each stimulated increased MCC velocities. Forskolin, VIP, and isoproterenol all elevate [cAMP], and stimulate CFTR-dependent submucosal gland secretion in many species including humans and ferrets but via somewhat different mechanisms and efficacies. The MCC responses to these three agonists are compared in Fig. 4. The mean (25–45 min) MCC velocities to each agonist were as follows: 10 M forskolin: 14.6 ± 3.1 mm/min, n = 10, Fig. 4A; 1 M VIP: 10.2 ± 2.2 mm/min, n = 5, Fig. 4B; and 10 M isoproterenol: 15.0 ± 1.9 mm/min, n = 5, Fig. 4C. The MCC velocities increased more gradually than did those for carbachol and also showed a gradual decline after peaking at 25–35 min (10–20 min after stimulation).

Measurement methodology gave consistent results when repeated by the same or different scorers. To determine to what extent the variation in the above results might be caused by the scoring process itself, three experiments were selected for repeat measurements by the initial scorer and then by an independent scorer who was blinded to the results of the other scorer (Fig. 5).

Phenylephrine is an α1-adrenergic, [Ca2+]i-elevating agonist that shows marked species differences in its ability to stimulate gland secretion. It has minimal effects in humans, sheep, or pigs (32) but strongly stimulates tracheal submucosal gland secretion in cat (42) and ferret (12). In ferrets, phenylephrine is less potent than carbachol but achieves the same efficacy at a concentration of 10 M. When applied after the 15-min basal period, 10 M phenylephrine caused a rapid increase in MCC, but in contrast with the sustained response to carbachol, the MCC velocity increased more slowly to peak at 30 min and then declined (Fig. 2B). The MCC velocity just before addition of phenylephrine was 2.0 ± 0.8 mm/min; it increased to an average of 15.3 ± 2.2 mm/min for the 25- to 45-min time period (P < 0.01, n = 5).

Carbachol-stimulated MCC velocity showed a steep dose-response relationship. A limited set of dose-response experiments was carried out for carbachol using concentrations from 0.1 to 10 M (Fig. 3, A and B). Under the conditions of our
Low concentrations of carbachol and isoproterenol interacted complexly to stimulate MCC. Synergy among agonists is said to occur if their combination yields a response larger than their expected additive effects. Potentiation is said to occur if an agent, which does not cause an effect on its own or has had its effect blocked, causes an increased response of a different agonist added later. Synergy is seen in gland secretion in several species, including humans, when low levels of agents that increase $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$ are combined (12, 13). Potentiation occurs with human sweat secretion when methacholine stimulation precedes a $\beta$-adrenergic cocktail (69).

We tested for interactions among agonist stimulation of MCC by combining 100 nM carbachol with 300 nM isoproterenol in three ways: simultaneous application (Fig. 6A), isoproterenol first followed 20 min later by the combination (Fig. 6B), and carbachol first followed 20 min later by the combination (Fig. 6C). Agonist concentrations were chosen to match those used in the studies of synergy with ferret submucosal glands (12). At these concentrations, the agonists do not increase MCC when used alone. Simultaneous application caused a small and nonsignificant increase in MCC from 0.8 to 2.2 $\pm$ 1.7 mm/min (Fig. 6A, $n = 3$), with a suggestion that stimulation might just be at threshold, since only one of three tracheas tested showed a response. However, when either of the agonists was added 20 min before the addition of the second agonist, the addition of the second agonist now produced much larger increases in MCC (Fig. 6, B and C, $n = 3$), and the order of presentation mattered in the same way that it did for gland secretion, with early addition of carbachol giving a larger response (see Ref. 12). Neither increase reached significance when comparing the mean values for the 15- to 35- and 50- and 65-min periods, but the combined data from six tracheas were significant (Fig. 6, B and C, $P < 0.05$).

![Fig. 4. Responses of MCC rate to agonists that elevate intracellular cAMP concentration ([cAMP]). A: response to 10 $\mu$M forskolin ($n = 10$, $P < 0.01$, ANOVA). B: response to 1 $\mu$M VIP ($n = 5$, $P < 0.05$, ANOVA). C: response to 10 $\mu$M isoproterenol ($n = 5$, $P < 0.01$, ANOVA).](http://ajplung.physiology.org/)

![Fig. 5. Scoring consistency of MCC. One experiment for each of 3 different agonists was chosen by the primary experimenter for rescoring by a second observer blinded to the original results but instructed in the criteria for scoring. The selected experiments had MCC values close to the mean of all experiments for that agonist. Open symbols and dashed lines were repeat measurements by the same scorer at different times. Closed triangle and solid line is the measurement of a second scorer who was blinded to the other results. A: different measurements of a single experiment testing the effects of 1 $\mu$M carbachol. B: a 2nd experiment scored as in A. C: a 3rd experiment with 10 $\mu$M isoproterenol, scored as in A and B.](http://ajplung.physiology.org/)
Inhibition of Cl− transport with bumetanide reduced MCC velocities more than HEPES replacement of HCO3−. Fluid secretion from both surface epithelia and glands depends on anion-mediated processes and can be inhibited by interfering with those processes. We inhibited Cl− secretion with bumetanide, HCO3− secretion with HEPES replacement, and combined the two treatments to study their effects on MCC stimulated by carbachol (Fig. 7A) or VIP + forskolin (Fig. 7B). Bumetanide inhibited MCC more effectively than HEPES for both agonists but especially so for MCC stimulated by VIP + forskolin, where it essentially abolished MCC. The summary results (Fig. 8A) show that carbachol-stimulated MCC was significantly decreased by bumetanide but not HEPES and the combination was the same as bumetanide alone. Inhibition of MCC stimulated by VIP + forskolin showed the same pattern (Fig. 8B) except that the effect of bumetanide was much more pronounced.

Agonists that elevate [cAMP], are much less effective than [Ca2+]i-elevating agonists in stimulating glandular secretion rates but have similar efficacy for stimulating increases in MCC velocities. Prior studies of ferret submucosal glands established stable, maximal secretion rates in response to five agonists: VIP, isoproterenol, forskolin, carbachol, and phenylephrine (12), and those data were used to inform the present studies of MCC. In Fig. 9A, the data from that study and the data from the present study were combined to construct a graph in which each point is jointly determined by the gland secretion velocity (x-axis) and the MCC velocity (y-axis) in response to five different agonists. The main finding was a discordance between the efficacy of agonists that elevated [cAMP], or [Ca2+]i, in stimulating gland secretion vs. MCC. As shown in Fig. 9A, the [Ca2+]i agonists were more effective in stimulating gland secretion than were the [cAMP],-elevating agonists, but they had similar effects on MCC. The mean level of gland secretion for the 3 [cAMP],-elevating agents was 252 pl·min−1·gland−1 (open symbols, x-axis) vs. 1,163 pl·min−1·gland−1 for [Ca2+]i-elevating agents (closed symbols, x-axis), a 4.6-fold difference (12). By contrast, the [cAMP], agents stimulated an average MCC velocity

Fig. 6. Synergy experiments. For each condition, plots show 3 individual experiments (open symbols and dashed lines) and the mean value (solid circle and line). A: response to combined agonists added simultaneously. B: response to combined agonists following 20 min isoproterenol. C: response to combined agonists following carbachol. Used singly, these agonists did not increase MCC velocity, but the combinations were effective and the order of addition mattered.

Fig. 7. Inhibition of MCC velocity by anion transport inhibitors. Four conditions were compared for each of 2 agonists: control (normal Krebs bath), 100 µM bumetanide, replacement of HCO3− with HEPES, and the combination of HEPES + bumetanide. In each condition, the altered bath solution was added ~15 min before time 0 and was present throughout the experiment. A: stimulation with 1 µM carbachol (n = 7). B: stimulation with 1 µM vasoactive intestinal peptide (VIP) and 10 µM forskolin (n = 6).
Inhibition of MCC velocity by anion transport inhibitors: summary data. Each bar shows MCC velocities (means ± SE) for the conditions shown. MCC in each trachea is based on the average of the last 3 time points (35–45 min) for each of the 4 conditions shown. A: stimulation with 1 μM carbachol alone and with anion transport inhibition. Conditions differed overall, P < 0.01, Kruskal-Wallis, as did post hoc comparisons P < 0.01, Mann-Whitney U-test. B: stimulation with 1 μM VIP + 10 μM forskolin alone and with anion transport inhibition. Conditions differed overall, P < 0.001, Kruskal-Wallis, as did post hoc comparisons P < 0.01, Mann-Whitney U-test.

Comparison of gland secretion rates and MCC velocities using different agonists and anion inhibitors. In Fig. 9B, the MCC velocity data from Fig. 8 were compared with previously obtained gland secretion rates that used identical conditions of stimulation and anion transport inhibitors (12). As in Fig. 9A, each point is jointly determined by the gland secretion rate (x-axis) and MCC velocity (y-axis) with type of agonist and ion transport inhibitors indicated. These provide a separate set of comparisons between secretion rates and MCC velocities and also reveal interaction effects.

DISCUSSION

These experiments test the hypothesis that the volume and properties of glandular mucus influence MCC velocity. We tested five agonists, two of which were also tested after inhibition of Cl⁻ or HCO₃⁻ transport. The agonists and inhibitors were the same as those used in a prior study of ferret tracheal gland secretion (12), allowing us to distinguish the volume of secreted mucus from other factors affecting MCC velocities (Table 1). Because each agonist produces only part of the effect expected from the release of multiple transmitters from airway intrinsic neurons (67), their contributions could be dissected. All five agonists stimulated both gland secretion and MCC but discordantly (Fig. 9A). For example, phenylephrine was seven times more effective than isoproterenol in stimulating mucus secretion, but the two agonists stimulated similar MCC velocities. The secretory pathways responsive to [cAMP], elevation are CFTR dependent, and their unexpected effectiveness on MCC is of special interest because it could mean that the mucus they produce is more transportable (10, 18, 20, 41, 43). However, MCC velocity depends on factors beyond the quality and quantity of the mucus blanket, so it is also important to consider how agonists affect those factors (Fig. 10).

CBF (49, 53, 55) is stimulated in various species by ACh or carbachol (50, 51), isoproterenol (34, 63), forskolin (9, 52, 71), and VIP (36, 48). However, ferret tracheal epithelium lacks functional VIP receptors (35), so CBF and other epithelial...
processes (see below) may play no role in the VIP-stimulated MCC increases we observed. In contrast with the other agonists, phenylephrine decreased CBF in rabbit and human nasal cells (21, 39, 40, 74). To our knowledge, a phenylephrine effect on ferret tracheal CBF has not been reported.

Surface epithelial anion-mediated fluid secretion is increased by agents that increase [Ca²⁺]. Airway epithelial cells in many species possess muscarinic and nicotinic ACh receptors (67), and cholinergic stimulation transiently increases anion secretion from airway epithelia (2, 4, 23). Agents that elevate [cAMP]ᵢ also increase surface fluid secretion (58, 66). Although VIP increases anion secretion from human airway epithelia (62), ferret tracheal epithelium, as noted above, lacks functional VIP receptors (35), so VIP-stimulated increases in MCC velocity might have come entirely from gland secretion. Phenylephrine had no effect on ion transport in rabbit trachea but neither did cholinergic or β-adrenergic agents (25). We found no reports of phenylephrine effects on epithelial anion transport in the ferret trachea.

Alteration of epithelial fluid absorption is an additional way to alter MCC velocity. After initially increasing anion secretion, cholinergic stimulation inhibits epithelial Na⁺ (and therefore fluid) absorption (2–4). Indeed, in many systems where epithelial sodium channel (ENaC) is expressed, elevating [Ca²⁺]ᵢ inhibits it (19). Thus it is possible that the increases in MCC velocity produced by carbachol and phenylephrine are aided by decreases in epithelial fluid absorption. By contrast, elevation of [cAMP], in many systems increases ENaC activity (24, 45, 65) at least in part by translocating ENaC to the membrane via PKA and protein tyrosine kinase mechanisms (72). This would be expected to reduce MCC velocity.

**Inhibitors of epithelial Cl⁻ and HCO₃⁻ transport reduced MCC velocity in an agonist-dependent way.** Anion transport inhibitors such as bumetanide reduce the volume and properties of fluid secretion from both glands and surface epithelial, without affecting CBF (8). We observed unexpected interactions among agonists and anion transport inhibitors. For carbachol, anion transport inhibitors produced comparable effects on MCC velocity and glandular mucus secretion rates (Fig. 9B and Table 2), with bumetanide inhibiting each by 68–70% and HCO₃⁻ replacement by 32–42%. However, after VIP + forskolin stimulation, inhibition of gland secretion and MCC were discordant. Gland mucus secretion rates were equally reduced by bumetanide or zero HCO₃⁻ (12), while in marked contrast, MCC was abolished by bumetanide but reduced only by 15% with zero HCO₃⁻. Said differently, the inhibitory effectiveness

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**Table 1. Gland volume secretion and MCC velocities for ferret and pig**

<table>
<thead>
<tr>
<th>Agonists</th>
<th>Ferret Gland Secretion</th>
<th>Ferret MCC</th>
<th>Pig Gland Secretion</th>
<th>Pig MCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh (1 μM)</td>
<td>Ref. 12</td>
<td>0.98 ± 0.14</td>
<td>19.1 ± 3.3 (10)</td>
<td>Ref. 31</td>
</tr>
<tr>
<td>ACh (100 μM)</td>
<td>1.35 ± 0.33</td>
<td>15.3 ± 2.4 (5)</td>
<td>1.04 ± 0.51</td>
<td>—</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>0.34 ± 0.07</td>
<td>10.2 ± 2.2 (5)</td>
<td>1.69 ± 0.22</td>
<td>—</td>
</tr>
<tr>
<td>Forskolin</td>
<td>0.23 ± 0.01</td>
<td>14.6 ± 3.1 (10)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>0.183 ± 0.09</td>
<td>15.0 ± 1.9 (5)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>VIP</td>
<td>0.35 ± 0.1</td>
<td>17.6 ± 1.3 (5)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are means ± SE; numbers in parenthesis are number of animals tested. Values for gland mucus secretion are nanoliters per minute per gland. Values for mucociliary clearance velocities are millimeters per minute and are based on the average of the velocities taken at 5-min intervals from t₀ to t₅ (see MATERIALS AND METHODS). Dash indicates experiments were not done. ACh, acetylcholine; Fsk, forskolin; MCC, mucociliary clearance; VIP, vasoactive intestinal peptide. *Peak value to 10 μM carbachol, stable secretion value was approximately 1/3 of the peak value.

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**Fig. 10. Coordinated neural effects on MCC.** A: schematic overview of multiple effects on MCC from airway intrinsic neurons. Each of the 5 agonists tested in this article are hypothesized to produce part of the effects expected from the release of cholinergic, adrenergic and peptide transmitters from airway intrinsic neurons under the control of vagal efferents and (not shown) sensory-motor neurons and nonneural humoral factors (67). Where they have been tested, most of these agonists also affect the other 3 components of mucociliary clearance, causing increased ciliary beat frequency, increased anion (and hence fluid) secretion from surface epithelia, and decreased ENaC-mediated Na⁺ (and hence fluid) absorption. B: cartoon of the in situ relations among the 4 components shown in A. ASL, airway surface liquid.
ratio for bumetanide/HEPES was ~1 for glandular mucus secretion (12) vs. 6.6 for MCC velocity (Fig. 8B and Table 2).

These ferret MCC results were opposite to those in pig tracheas, where cholinergically stimulated MCC was inhibited more by zero HCO$_3^-$ than by bumetanide (14). The main difference was the effectiveness of bumetanide, which inhibited ferret MCC by 70% but had no effect in the pig, whereas zero HCO$_3^-$ inhibited MCC approximately equally (42% in ferret vs. 35% in pig). The experiments differed in several ways: most obviously in the species but also in agonist concentrations (100-fold higher in pig) and a longer measurement period in pig. Trout et al. (61) observed that bumetanide pretreatment substantially elevated mucus liquid HCO$_3^-$ concentrations. These elevated HCO$_3^-$ concentrations could have ameliorated the inhibitory actions of volume reduction on MCC by potentially improving mucus viscosity and transportability (10, 18, 20, 41, 43) despite decreases in ASL volume (14). Cooper et al. (14) did not use cAMP-elevating agonists in their pig experiments, and these produced our most surprising results: cAMP-stimulated MCC was unaffected by removing HCO$_3^-$ but was abolished by bumetanide. It may not be coincidental that basal MCC in the pig was inhibited more effectively by bumetanide than by zero HCO$_3^-$ (14).

**Limitations of ex vivo experiments.** To evaluate the extent to which ex vivo conditions might compromise the results, we compared our MCC velocities with results from prior in vivo and in situ experiments. In vivo, sodium iodide radiation detectors monitored radiolabeled albumin placed on the dorsal trachea of anesthetized, supine ferrets (38). A mean basal MCC velocity of 1.0 mm/min was maintained for 5 h and was approximately doubled by a maximal dose of an anticholinesterase. In situ, the ventral trachea was cut and tantalum powder movements visualized on the dorsal trachea of anesthetized, supine ferrets from birth to >4 wk (64). During this time, the submucosal glands develop, and the trachea becomes ciliated and its length increases from 2.2 to 5.5 cm (37). Mean MCC velocities increased from 0 to 11 mm/min at 4 wk, where they remained (64). These transport velocities are in reasonable agreement with the values we observed after stimulation and reinforce the view that intact airways receive tonic excitatory input (68).

Few prior studies have been done on ex vivo ferret MCC velocity, and these have only studied basal MCC. An MCC of 9.5 ± 3.4 mm/min was measured for bits of ferret mucus placed on the tracheal surface after prior submersion of the segment for 30 min at 38°C in Krebs-Henseleit (1). In the only study of MCC in CF ferrets, movements of 1 μm fluorescent beads were measured in the upper third of tracheas from control and CF ferret tracheas (3–8 mo of age) (59). The segments were divided longitudinally and placed in a humid chamber on filter paper saturated with 37°C DMEM. Control MCC was ~2–5 mm/min, and CF MCC was ~0–2.5 mm/min (n = 6 pairs). Regardless of the absolute values, this large difference indicates the importance of CFTR-mediated processes to MCC.

**Summary and conclusions.** The ex vivo ferret trachea, under the conditions used here, supports basal MCC only briefly. The lack of central innervation and the absence of ASL that would normally flow onto the distal trachea from the rest of the lung result in the trachea being swept clear of ASL in a caudal to proximal direction. Basal secretion of fluid by the trachea is not sufficient to offset the losses to mucus transport, fluid absorption, and evaporation. All agonists were similarly effective in restoring MCC velocities to values observed in vivo for anesthetized ferrets, suggesting that the lack of innervation was probably the most important factor between the in vivo and ex vivo results. The similarity among agonists in stimulating MCC was in contrast with large differences in their effectiveness in stimulating gland mucus secretion, where agents that act primarily via increasing [Ca$^{2+}$], are much more effective than those that primarily increase [cAMP]. The disparity might arise for multiple reasons, including effects of the agonists on other components of MCC (Fig. 10) or greater transportability of mucus secreted by elevating [cAMP], vs. mucus secreted by elevating [Ca$^{2+}$], which could compensate for lower fluid secretion associated with the latter (10, 14, 43, 73). Regardless, these methods and the parametric data generated from control animals provide a useful basis for comparing stimulated MCC in control and CF ferrets.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise are declared by the author(s).
REFERENCES


6. Ballard ST, Trout L, Mehta A, Inglis SK. PKA induces Ca2+ release and en-...


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