Regulation of the depth of surface liquid in bovine trachea


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Wu, D. X.-Y., C. Y. C. Lee, S. N. Uyekubo, H. K. Choi, S. J. Bastacky, and J. H. Widdicombe. Regulation of the depth of surface liquid in bovine trachea. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L388–L395, 1998.—The luminal surface of airways is lined by a thin film of airway surface liquid (ASL). Physiological regulation of the depth of ASL has not been reported previously. In this paper, we have used low-temperature scanning electron microscopy of rapidly frozen specimens of bovine tracheal epithelium to demonstrate alterations in the depth of ASL in response to the cholinergic agonist methacholine. We first established that methacholine selectively stimulated airway glands, with maximal secretion at −2 min and a return to baseline within ~5 min. A 2-min exposure to methacholine increased the depth of ASL from 23 to 78 µm. Thereafter, depth decreased linearly with time, reaching 32 µm at 30 min. The initial increase in depth was blocked by bumetanide, an inhibitor of active chloride secretion, whereas the slow decline back to baseline was inhibited by amiloride, a blocker of active sodium absorption. We conclude that the methacholine-induced changes in ASL depth reflect transient gland secretion followed by liquid absorption across the surface epithelium.

MATERIALS AND METHODS

Bovine tracheae were obtained from a nearby slaughterhouse. In some cases, within minutes of removal from the animal, pieces of trachea were rapidly frozen by immersion in liquid nitrogen. Other tracheae were transported to the laboratory in ice-cold, preoxygenated Krebs-Henseleit solution (KHS) of the following composition (in mM): 120 NaCl, 25 NaHCO₃, 5 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 6 glucose, and 0.05 phenol red. The trip from the slaughterhouse took 1–2 h. Once in the laboratory, epithelial sheets (~25 cm²) were dissected away from the underlying tissues and cut into individual 1-cm² squares. These were rinsed briefly in KHS with or without amiloride. They were then pinned out, mucosal side up, on sponges saturated with KHS and maintained in a warm (37°C) humidified atmosphere of 95% O₂-5% CO₂. The KHS in the sponges contained methacholine alone (10⁻⁵ M), methacholine plus amiloride (10⁻⁵ M), methacholine plus bumetanide (10⁻⁴ M), or no drugs. After set periods, the tissues were rapidly frozen when a cryoprobe with a flat polished copper surface, precooled to the temperature of liquid nitrogen (~196°C), was brought into contact with the mucosal surface. From this point on, the samples were maintained at less than ~180°C and processed as previously described for rapidly frozen pieces of lung (3).

First, liquid nitrogen was poured over the probe tip with a flat polished copper surface, precooled to the temperature of liquid nitrogen (~196°C), was brought into contact with the mucosal surface. From this point on, the samples were maintained at less than ~180°C and processed as previously described for rapidly frozen pieces of lung (3). First, liquid nitrogen was poured over the probe tip and adjacent tissue. The frozen tissue was then stored in liquid nitrogen. Later, the tissue was trimmed under liquid nitrogen with a circular dental saw and then fitted into a miniature vise with its serosal surface facing one jaw and its mucosal surface facing the other. Otherwise, orientation of tissue sheets within the vise was completely random (i.e., there was no set relationship between the jaws of the vise and the oral, aboral, left or right sides of the tissue). The vise with its mounted tissue was transferred via a vacuum air lock to a cryochamber attached to the microscope (AMRay Biochamber; AMRay, Bedford, MA). The tissue was viewed with a dissecting microscope while a precooled knife fractured it at right angles to the epithelial surface. If necessary, fracturing was repeated until a clean, smooth fracture plane perpendicular to the epithelial surface was obtained. Some frozen,
hydrated tissues were coated with gold at this point. Others were first radiant etched for periods of up to 1 min using a tungsten filament 10 mm from the tissue, which was heated to dull red. Once coated with gold, tissues were mounted on a scanning electron microscope stage cooled with a high-pressure Joule-Thompson nitrogen refrigerator (less than −180°C). The stage was adjusted until the electron beam was parallel to the epithelial surface. Stereo pair electron micrographs were made at a tilt difference of 10° (5) using a lanthanum hexaboride electron source at 10 kV and an electron beam of 5 × 10−12 A (Faraday cup measurement). Images were recorded on Polaroid 55 positive-negative 4 × 5-in. sheet film (Polaroid, Cambridge, MA). Starting near the center of the fracture plane (total length = ~5 mm), 10 estimates of the depth of ASL were made at intervals of 10 µm. The field of vision was then moved 100 µm to the right along the fracture plane, and a further 10 estimates of ASL depth were made. In total, 10 groups of 10 estimates were made. The average of all 100 estimates was taken as the ASL depth for that specimen.

Ussing chambers were used to determine the general viability of the tissues and the types of transepithelial ion transport present. Sheets of tissue were mounted between lucite half-chambers with an exposed surface area of 1.3 cm². Warm (37°C), oxygenated KH5 was circulated across both faces of the tissue using gas lift oxygenators. Transepithelial potential difference (PD) was sensed by agar bridges connected via calomel half-cells to a high-impedance electrometer. Agar bridges at the backs of the half-chambers were connected to a voltage clamp (University of Iowa Bioengineering, Iowa City, IA), which was used to pass current to clamp the PD to zero. The resulting short-circuit current (Isc), which is equal to the sum of the active ion transport processes generating the PD (27), was displayed continuously on a chart recorder. Amiloride (10−5 M, mucosal bath) was first used to inhibit active sodium absorption. Methacholine and forskolin (both at 10−5 M in both baths) were then added in random order, the latter being used as a stimulator of adenosine 3',5'-cyclic monophosphate-dependent chloride secretion across the surface epithelium (13). Finally, chloride secretion was inhibited with diphenylamine-2-carboxylate (2 × 10−5 M, serosal bath). Transepithelial electrical resistance (Rw) was determined every 20 s from the current needed to clamp PD from zero to a constant value (0.5–2.0 mV) for 200 ms.

Gland secretion was measured by the “hillocks” technique (4, 6). A lucite ring held a piece of epithelium mucosal side up over the top of a lucite cylinder. The surface of the epithelium was coated with tantalum dust and viewed with a dissecting microscope. Warm, oxygenated KH5 was introduced into the lucite cylinder on the serosal face of the tissue and was maintained at 37°C by a water jacket. Gland secretions were revealed as upswellings (hillocks) in the tantalum coating.

Gland openings were visualized by epillumination of unfixed tissue sheets, by staining of living tissue with neutral red (26), or by staining whole mounts of epithelium with alcian blue-periodic acid Schiff (PAS; see Ref. 25). Total numbers in fields of 20–70 mm² were counted. All three methods gave similar values for the numbers of gland openings per unit area of epithelium. Goblet cells were clearly visible in the whole mounts stained with alcian blue-PAS.

The volume of glands per unit area of airway surface was obtained as follows. Sheets of epithelium were fixed in 4% formaldehyde in saline and embedded in paraffin. Cross sections were stained with hematoxylin and eosin, and one end of the section was displayed at ×130 magnification on a video screen. The areas occupied by profiles of surface epithelium and glands were traced. The slide was moved to display the adjoining length of section on the screen, and again the surface epithelium and gland profiles were marked. This process was repeated until the entire length of section was scanned; sections were ~1 in. long and were covered by ~10 video screens. The length of epithelium on each transparency was determined in inches using a cartographic measuring wheel. The total area of gland profiles on each transparency was determined gravimetrically in square inches. From the known magnification on the video screen, the ratio of square inches of gland per inch of epithelium on the transparency was converted to square millimeter per millimeter on the section. This is the same as the ratio of gland volume to airway surface area in microliter per square centimeter times 10−2.

Data are expressed as means ± SE. Significance of differences between means was assessed by Student's paired t-test, with P < 0.05 considered statistically significant.

RESULTS

As assessed from their electrical properties, tissues had good viability on arrival at the laboratory, and this was not compromised by dissection of the epithelium away from the underlying tissues. Figure 1, for instance, shows Isc across two sheets of epithelium from the anterior portion of the same trachea. In one sheet, the underlying collagen rings had been dissected away, and, in the other sheet, the rings were left in place. Baseline Isc values were similar (~150 µA/cm²) and were inhibited ~65% by amiloride. Both sheets showed sustained increases in Isc of ~40 µA/cm² in response to forskolin. Methacholine transiently stimulated Isc across the dissected epithelium but not when the submucosal layers were intact. Methacholine receptors are on the
basolateral membrane of airway epithelium and glands (2). Thus the failure of this agent to affect undissected tissues may reflect the diffusional barrier provided by the cartilage rings, which are ~0.5 cm thick. Finally, dihydroxylamine-2-carboxylate markedly inhibited the amiloride-insensitive $I_{sc}$ in both tissues. Baseline $I_{sc}$ and $R_{te}$ of epithelial sheets dissected from the anterior portion of the trachea were $84 \pm 33 \mu A/cm^2$ and $92 \pm 30 \Omega \cdot cm^2$, respectively (means ± SD, n = 10).

To test whether the $I_{sc}$ response to methacholine represented an effect on surface epithelium or glands, we compared electrical properties of epithelial sheets dissected from the anterior cartilaginous or posterior membranous portions of the trachea. The anterior portion had $1.60 \pm 0.07$ gland openings/mm² (25 fields from 4 tracheas). The gland density in the posterior membranous portion was $0.82 \pm 0.05$ openings/mm² (n = 22 fields from 3 tracheas). Not only were there fewer gland openings in the posterior portion, but the glands were smaller. Thus total gland volume was $17.7 \pm 1.2 \mu l/cm²$ (5 sections from 2 tracheas) in the anterior portion of the trachea and $5.3 \pm 1.2 \mu l/cm²$ in the posterior portion (4 sections from 2 tracheas). Epithelium from the anterior portion of the trachea showed maximal increases in $I_{sc}$ of $18.2 \pm 3.6 \mu A/cm^2$ (n = 10) in response to methacholine. In about one-half of the tissues, the response to methacholine was sustained for at least 15 min. In the others, it was maximal at ~2 min and declined to baseline after ~5 min. By contrast, epithelial sheets from the posterior membranous portion of the same trachea showed no or little response to methacholine (maximal increase = $1.7 \pm 1.1 \mu A/cm^2$, n = 10). Baseline $I_{sc}$, $R_{te}$, and the forskolin-induced increase in $I_{sc}$ (~30 µA/cm²) were not significantly different for epithelium from the anterior or posterior portions of the trachea.

Induction of gland secretion by methacholine was confirmed by the hillocks technique (Fig. 2). Within 1 min of dissection, tissues were mounted and coated with tantalum. A few small hillocks appeared shortly after coating. However, if the saline on the mucosal faces of the tissues was replaced with fresh medium at 7.5 min, this caused no further hillock formation (Fig. 2, A and B). By contrast, if methacholine was added to the serosal bath, preexisting hillocks grew in size, and new hillocks appeared (Fig. 2, C and D). The maximal number of hillocks induced by methacholine in sheets of epithelium from the anterior portion of the trachea ($0.96 \pm 0.03$ per mm²; n = 5) was ~60% of the number of duct openings revealed by histological techniques, suggesting that not all glands responded to methacholine. Methacholine had no effect on the numbers of goblet cells or their intensity of staining with PAS-alcian blue (data not shown).

Figure 3A shows a low-magnification electron micrograph of an unetched fracture plane revealing the characteristic pseudostratified appearance of airway epithelium. Cilia are apparent, and the mucosal surface of the cells is covered by a layer of liquid 10–15 µm in depth. Figure 3B shows a higher magnification view of a cross-fracture through another unetched tissue. Cilia are dearly visible at this magnification, and this particular specimen contained several mature goblet cells. Etching of the fracture surface made the cilia more obvious and frequently revealed two layers in the ASL, with the layer closest to the epithelium having the larger ice-crystal voids (Fig. 3C). The depth of ASL in control tissues studied in the laboratory was $23 \pm 3 \mu m$ (n = 10 tracheas). This was similar to the depth of ASL in a tissue frozen at the abattoir (19 ± 3 µm).

In two experiments, depth of ASL was determined at 1, 2, 5, 10, and 30 min of exposure to methacholine. In
both experiments, the depth of ASL was greatest at 2 min and declined approximately linearly between 2 and 30 min. In further experiments, depth was measured at 2 and 30 min of exposure. Figure 4 shows three epithelial sheets illustrating the effects of methacholine on the appearance of ASL. In unstimulated tissues, frozen within 30 s of mounting on the sponge (Fig. 4A), the ASL was not much deeper than the length of the cilia (~6 µm). After 2 min of exposure to methacholine, ASL depth increased approximately sevenfold, and...
radiant etching revealed the presence of two layers in
the ASL (Fig. 4B). After 30 min of exposure to methacholine, the
depth and appearance of ASL differed little from control (Fig. 4C).

Figure 5 shows all 100 measurements of ASL depth for two
tissues from the same trachea, one untreated and the other exposed
to methacholine for 2 min. The marked regional variation in depth is
due to folding of the surface epithelium. The average of all 100 esti-
mates was taken as the ASL depth for each particular specimen. Figure 6 shows that ASL depth, so determined, was increased by a 2-min exposure to methacholine in all 10 tracheas studied. Again, in all tracheas, depth declined between 2 and 30 min of exposure (Fig. 6). When data from all 10 tracheas were combined, a 2-min exposure to methacholine increased the depth of ASL from $23 \pm 6$ to $78 \pm 9 \mu m$. Between 2 and 30 min of exposure, ASL depth declined to $32 \pm 5 \mu m$.

Fig. 4. Effects of methacholine on depth of airway
surface liquid. a: control tissue not exposed to methacholine.
b: 2-min methacholine exposure. Putative sol and mucous gel are clearly visible. c: 30-min exposure. Tissues were radiant etched for 20 s to 1 min. Scale bar = 20 µm.
Figure 7A shows that amiloride had no effect on the initial increase in depth seen during the first 2 min of exposure to methacholine but abolished the decrease in depth between 2 and 30 min. By contrast, bumetanide (10^{-2} M in the serosal bath) markedly inhibited the initial increase in depth (Fig. 7B).

Untreated time-control tissues from two tracheas showed small variable changes in depth between 0 and 30 min (from 11.0 to 16.5 µm and from 14.2 to 10.9 µm). Increases in depth in response to a 2-min exposure to methacholine were normal in tissues from the same tracheas.

DISCUSSION

Our studies are the first to demonstrate physiological regulation of the depth of ASL. Specifically, we show that stimulation of submucosal gland secretion causes a rapid threefold increase in depth followed by a slow decline, which is inhibited by amiloride, a blocker of active absorption of sodium across the surface epithelium.

The depth of ASL in living tissues has been measured with microelectrodes or by light microscopy. When cultures of canine bronchial epithelium were grown with no medium added to their mucosal surfaces ("air interface feeding"), microelectrodes revealed an ASL depth of 15 µm (12). Rahmoune and Shephard (20) poked microelectrodes through a small window cut in the wall of guinea pig tracheas in vivo and recorded the distance between the surface of ASL (electrical circuit formed) and the apical membranes of the cells (electrical interference). When the animals breathed air in which the water vapor pressure favored condensation, the depth of ASL averaged 87 µm. Breathing dry air reduced the depth to zero in ~15 min. In vitro, microelectrodes measured an ASL depth in isolated guinea pig tracheas of ~200 µm when the mucosal surface was exposed to warm humidified air. The depth decreased when dry air was blown over the tracheal surface (19, 23). Seybold et al. (22) viewed the surface of sheep tracheal epithelium with epi-illuminating dark-field and bright-field optics. Using a displacement transducer with a resolution of 0.5 µm, they determined the position of the epithelial surface under bright-field optics and the air-liquid interface using the dark-field mode. They found the ASL depth to be 35–50 µm. The tracheas were exposed to 10^{-5} M acetylcholine or epinephrine, and the depth of ASL was determined after 20 min. Next, tissues were exposed to 10^{-4} M of either drug, and the depth was again measured after 20 min. Finally, tissues were exposed to 10^{-3} M mediator for 20 min. Neither drug, at any concentration, altered ASL depth.

The failure of Seybold et al. (22) to detect changes in the depth of ASL in response to autonomic agonists is surprising given that there are a number of processes that effect liquid flows across airway epithelium, and some of these are under autonomic control. For instance, active absorption of sodium across human surface epithelium in vitro removes luminal liquid at ~5 µl·cm^{-2}·h^{-1} (10), a rate that would decrease the depth of ASL by ~1 µm/min. Neurohumoral agents can induce active secretion of chloride across human and dog tracheal epithelium, adding liquid to the lumen at ~3 µl·cm^{-2}·h^{-1} (10, 31). Chloride secretion across bovine tracheal epithelium can also be stimulated by several neurohumoral agents (14). Maximal secretion from airway glands in cats, induced by cholinergic or α-adrenergic agents, is ~10 nl·min^{-1}·gland^{-1} (18, 26). Depending on species and airway region, gland density varies from 1 to 10 per mm² (1, 25). Thus maximal gland secretion could be 60–600 µl·cm^{-2}·h^{-1} and should increase the depth of ASL by 10–100 µm/min. Finally, the marked expansion of goblet cell granules on discharge (29) could draw liquid from beneath the epithelium and also increase ASL depth.

We have previously established that low-temperature scanning electron microscopy of rapidly frozen tissues can be used to measure the depth of ASL (32). The advantage of this approach over other microscopic techniques is that shrinkage and some artifacts associated with processing of the tissues are avoided.
The tracheas used in these studies were kept in ice-cold saline for up to 2 h during their transfer from the slaughterhouse to the laboratory. The surface epithelium and glands were then dissected from the underlying cartilage. Ussing chamber studies, however, showed that the epithelium was in good health on arrival and was not damaged by dissecting away the underlying tissues. In fact, the values for $I_{sc}$ and $R_{te}$ reported here for dissected epithelium are comparable to those reported for sheets of intact bovine tracheal wall by other investigators (9).

The depth of ASL in control, unstimulated tissues was 23 µm. Even though these tissues were rinsed with saline before study, the depth of their ASL was reasonably close to that seen in tissues frozen immediately on removal from the animal (19 µm).

Cholinergic agents are potent stimulators of airway gland secretion in many species (9), but their effects on bovine airway glands have not been reported. Using the hillocks technique, we demonstrated cholinergically mediated gland secretion in bovine trachea. Alcian blue-PAS staining of epithelial sheets showed no change in the numbers of goblet cells stained or in their staining intensity. This is in agreement with a previous study indicating that airway goblet cells of most mammalian species are insensitive to cholinergic stimulation (28). Finally, methacholine transiently increased $I_{sc}$ across epithelium from the anterior portion of the trachea but had little effect on $I_{sc}$ across epithelial sheets from the posterior membranous portion of the trachea, a region containing comparatively few glands. Thus methacholine would seem to be a specific stimulator of gland secretion, and the small transient change in $I_{sc}$ induced by methacholine across the anterior portion reflects the time course of methacholine-stimulated gland secretion. In most tissues, this transient was maximal at ~2 min and essentially finished within 5 min, in approximate agreement with the time course of mediator-induced gland secretion reported by others (4, 8, 18, 26).

The maximal depth of ASL occurred at approximately the same time as the peak in gland secretion predicted from the methacholine-induced increase in $I_{sc}$. The increase in depth over the first 2 min of methacholine stimulation (~50 µm) corresponds to a volume flow of 150 µl·cm$^{-2}$·h$^{-1}$. The average density of glands in the anterior portion of bovine trachea is ~2 mm$^{-2}$. Maximal gland secretion in cats is ~10 nl·min$^{-1}$·gland$^{-1}$ (18, 26). With the assumption of similar flow rates in bovine airway glands, maximal secretion should be 120 µl·cm$^{-2}$·h$^{-1}$, in good agreement with the volume flow calculated from the change in depth. The liquid component of gland secretions is probably produced secondarily to active sodium-linked secretion of chloride (1, 33). Consistent with this, we found that the initial increase in depth was inhibited by bumetanide, a blocker of epithelial chloride secretion.

Between 2 and 30 min of methacholine exposure, ASL showed a decline in depth of ~40 µm. This decline was blocked by amiloride, thereby directly implicating osmotic gradients created by active absorption of sodium as the mechanism behind the depth decrease. Further support for this idea is the good quantitative agreement between transepithelial volume flows measured directly or estimated from the decline in ASL depth. Thus baseline liquid absorption across primary cultures of bovine tracheal epithelium is ~5 µl·cm$^{-2}$·h$^{-1}$ and is abolished by amiloride (S. Uyekubo, J. H. Widdicombe, and S. S. Miller, unpublished observation). The decline in depth from 75 to 30 µm between 2 and 30 min of methacholine exposure yields a similar value for liquid absorption across native tracheal epithelium of ~9 µl·cm$^{-2}$·h$^{-1}$.

Our results contrast with those of Seybold et al. (22), who failed to see an effect of acetylcholine on the depth of ASL in isolated sheep tracheas. However, their measurements of depth were made after a 20-min
exposure to drug. Our results in the bovine trachea suggest that gland secretion is transient and is followed by liquid reabsorption. Therefore, it is possible that Seybold et al. missed an initial transient increase in depth. We also note that the baseline depth of ASL was somewhat deeper (~50 µm) in the studies of Seybold et al. than in ours (~25 µm).

In both control and methacholine-stimulated tissues, radiant etching frequently revealed the presence of two layers. The layer closest to the epithelium had large ice-crystal voids, and we speculate that it corresponds to the periciliary sol. The upper layer with smaller voids may be the mucus gel. In control tissues, the depth of the putative sol was approximately the same as the length of the cilia (Figs. 3 and 4A), as would be expected for optimal mucus transport. Further experimental approaches are needed, however, to determine whether the regions of small and large ice-crystal voids do indeed correspond to the gel and sol.

In conclusion, our results establish that gland secretions can cause large and rapid increases in the depth of ASL in vitro, followed by a return to baseline effected by active absorption of sodium across the surface epithelium.

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2 Although the changes were not quantified, the depths of both the putative sol and gel increased after stimulation with methacholine (Fig. 4B). Thus, during stimulation, the putative sol became considerably deeper than the length of the cilia, suggesting that mucociliary clearance should be impaired at the height of gland secretion.