Optical method for quantifying rates of mucus secretion from single submucosal glands

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Joo, Nam Soo, Jin V. Wu, Mauri E. Krouse, Yamil Saenz, and Jeffrey J. Wine. Optical method for quantifying rates of mucus secretion from single submucosal glands. Am J Physiol Lung Cell Mol Physiol 281: L458–L468, 2001.—We describe an optical method to quantify single-gland secretion. Isolated tracheal mucosa were mounted at the air-Krebs interface and coated with oil. Gland secretions formed spherical bubbles that were digitally imaged at intervals, allowing rates of secretion to be calculated. We monitored 340 glands in 54 experiments with 12 sheep. Glands secreted basally at low rates (0.57 ± 0.04 nl·min⁻¹·gland⁻¹, 123 glands) in tissues up to 9 h postharvest and at lower rates for up to 3 days. Carbachol (10 μM) stimulated secretion with an early transient and a sustained or oscillating phase. Peak secretion was 15.7 ± 1.2 nl·min⁻¹·gland⁻¹ (60 glands); sustained secretion was 4.5 ± 0.5 nl·min⁻¹·gland⁻¹ (10 glands). Isoproterenol and phenylephrine (10 μM each) stimulated only small, transient responses. We confirmed that cats have a large secretory response to phenylephrine (11.6 ± 3.7 nl·min⁻¹·gland⁻¹, 12 glands), but pigs, sheep, and humans all have small responses (<2 nl·min⁻¹·gland⁻¹). Carbachol-stimulated peak secretion was inhibited 56% by bumetanide, 67% by HCO₃⁻ replacement with HEPES, and 92% by both. The distribution of secretion rates was nonnormal, suggesting the existence of subpopulations of glands.

carbachol; phenylephrine; cystic fibrosis transmembrane conductance regulator; lung disease; mucociliary clearance

SUBMUCOSAL GLANDS are a major source of the mucus that coats the luminal surface of cartilaginous airways. Submucosal glands are complex structures comprising multiple tubules that feed into a large collecting duct that narrows on its way to the airway surface (25). The tubules are lined with mucous cells and serous cells. Serous cells are predominant in the acini so that their watery secretions wash over mucous cells and then mix with mucins in the collecting duct before being expelled (24). Serous cell secretions are rich in antimicrobials and antioxidants that are important components of mucosal defense (3). In the genetic disease cystic fibrosis, gland malfunction may contribute to the genesis of airway infections. Cystic fibrosis transmembrane conductance regulator (CFTR), the protein that is defective in cystic fibrosis, is heavily expressed in gland serous cells (9). Cholinergic stimulation induces gland secretion in porcine bronchi that is driven by Cl⁻ and HCO₃⁻ (1, 17, 18, 37) and involves CFTR (2). If serous cells depend on CFTR for fluid secretion, as indicated by studies of primary cultures of gland cells (19, 42) and cell line models of serous cells (12, 26, 32), the resulting alterations in gland secretion might compromise mucosal defenses.

As a prelude to comparing submucosal gland function in normal and cystic fibrosis airways, we are developing methods to assess the function of individual glands. Functional data can then be combined with gland morphology obtained by other methods (26). Structural and functional studies need to be combined because glands become larger and more numerous in response to airway disease, and these changes must be considered when comparing secretion rates. In addition, individual glands vary in both size and cellular components (31) and may be differentially affected by disease. In prior studies of secretion rates of single glands, mucus was sampled with constant-bore micropipettes. These were either applied directly to the gland duct orifice (6, 10, 11, 22, 39) or used to collect bubbles of mucus that had formed under an oil coating (29). These methods are accurate but tedious and thus limit both the number of glands sampled and the minimal sampling interval. To allow rapid, frequent interval assessment of secretion rates in multiple, individually localized glands, we have modified the methods of Quinton (29) so that we can measure secretion rates optically with a digital video camera.

In this study, we applied these methods to tracheal submucosal glands of sheep. No adequate animal model of cystic fibrosis lung disease is presently available because CFTR-deficient mice do not develop airway disease. Fortunately, continuing improvements in cloning methods presage the development of other CFTR-deficient animals, some of which have airways more similar to humans. Sheep have particular advantages in this regard because they are suitable for cloning (4) and because prior studies of sheep airways indicate similarities with human airways (36), including a good complement of submucosal glands (5, 23).
However, there have been no prior functional studies of intact submucosal glands in sheep. In this study, we used our newly developed optical methods to quantify gland secretion in sheep. We discovered a marked species difference in response to α-adrenergic stimulation, document an extreme range of secretory rates across individual submucosal glands, and show that gland secretion in sheep, as in pigs (2, 16, 37), depends on both Cl⁻ and HCO₃⁻ transport.

METHODS

Animal tracheas were harvested <1 h postmortem from 16 wethers sheep (Suffolk-Rambouillet), 4 female pigs (Yorkshire), and 2 male cats, all adult. All animals had been killed with pentobarbital sodium injection after acute experiments unrelated to the present studies. Two pieces of human trachea were obtained as surgical trimmings from lung transplant donors. All tracheas were maintained until used in ice-cold Krebs-Ringer bicarbonate buffer (KRB) bubbled with 95% O₂-5% CO₂. The KRB composition was (in mM) 115 NaCl, 2.4 K₂HPO₄, 0.4 KH₂PO₄, 25 NaHCO₃, 1.2 MgCl₂, 1.2 CaCl₂, and 10 glucose (pH 7.4). Osmolarity was measured on a Westcor vapor pressure osmometer and was adjusted to ∼290 mosmol/l. To minimize tissue exposure to endogenously generated prostaglandins during tissue preparation and mounting, 1.0 μM indomethacin was present in the bath throughout the experiment unless otherwise indicated.

For each experiment, a tracheal ring of ∼1.5 cm was cut off, opened up along the dorsal (posterior) fold in ice-cold, oxygenated KRB, and pinned mucosal side up on a pliable silicone surface. Only the cartilaginous portion of trachea was used. The mucosa with underlying glands was carefully dissected from the cartilage and connective tissues and mounted in a 35-mm, Sylgard-lined plastic petri dish with the serosa in the bath (2-ml volume) and the mucosa in air. The dish was transferred to a temperature- and humidity-controlled chamber (Medical Systems, Greenvile, NY) and was gradually warmed to room temperature. The tissue surface was blotted dry and then further dried with a gentle stream of inert gas, after which 30–40 μl of water-saturated mineral oil were placed on the surface. The tissue was then warmed to 37°C at a rate of ∼1.5°C/min. Some secreting glands were observed at room temperature, and many more started secreting as the bath was warmed. The process of cleaning, drying, and oiling the epithelium did not appear to stimulate or inhibit secretion because similar secretion was observed in tissues or areas not so treated. “Basal” secretion refers to the secretion observed in otherwise unstimulated tissues.

Optical measures. The experimental setup is shown in Fig. 1A. For most experiments, the chamber was continuously superfused with warmed, humidified 95% O₂-5% CO₂ to minimize evaporation and to maintain bath pH at 7.4. The preparation was obliquely illuminated with a fiber-optic illuminator. The appearance of the secreted mucus

![Fig. 1. A: experimental setup. KRB, Krebs-Ringer bicarbonate buffer. B: schematic diagram showing formation of a spherical droplet (4/3πr³) of mucus at the mouth of a single-gland duct under oil. Arrows denote direction of liquid secretion. C: representative top and side views of mucus bubbles. Because of difficulties in placing the prism, we did not attempt to image the identical field. The two images are from adjacent areas in the same tissue, taken within 5 min of each other. D: control experiments showing stable volume over time for droplets of KRB in oil. Images are top views of ∼285- and ∼75-nl droplets at start (a) and end (b) of 3-h incubation. Scale bar, 0.5 mm.](http://ajplung.physiology.org/)

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droplets was strongly dependent on details of illumination that were adjusted empirically for each preparation. A digital video camera (Logitech) was mounted on one optical tube of a dissecting microscope. The image projected on the charge-coupled device sensor was captured as a bitmap representing an area \( \sim 6.25 \text{ mm}^2 \). The resolution was \( 640 \times 480 \) pixels, yielding \( \sim 49,000 \) pixels/mm\(^2\). Digital images were captured at intervals of 1–5 min using software supplied with the camera and were stored on disk for subsequent analysis using a modification of National Institutes of Health Image software (version 4.2; Scion, Frederick, MD). The captured image was calibrated using a 0.5-mm grid. The area from the perspective of the optical axis of the microscope of each droplet was then measured and converted to volume (V) using the spherical approximation \( V = \frac{4}{3} \pi r^3 \), where \( r \) is the radius. During analysis, we discovered that measurements in the leftmost 35 pixels of the image field were underestimated by the software package if measured with the freehand or circle tools. All other areas were accurate, as were measurements in this area made with rectangle or line measurement tools. If an image in the leftmost field was outlined with freehand or circular tools and the image was then moved out of the area, the measurement was again accurate.

When the surface was properly cleaned and dried, the volume of secreted bubbles was easily determined because they usually formed spheres that did not contact the surface except for continuity of the mucus at the gland opening. Nonspherical bubbles could usually be approximated by a sphere. The error of this approximation was proportional to the square root of the short axis/long axis (because this ratio is always <1, the rate calculation may underestimate the actual secretion rate). In some cases, the droplets obviously adhered to the surface and spread, and these were omitted from the analyses. With continued secretion, spheres from adjacent duct openings could merge, and in such cases, measurement was discontinued. A schematic diagram showing a mucous bubble forming above a gland is shown in Fig. 1B.

To assess the possibility that mucus bubbles that appeared to be spheres might actually be partially adherent, we visualized them from the side using a prism (Melles Griot, Irvine, CA).

Control experiments were done in pigs because sheep were no longer available. This revealed that, although the bubbles were clearly spherical (i.e., there was no flattening or elongation in the z-axis), many appeared to have contact angles <180° (Fig. 1C). To estimate the proportion of droplets with different contact angles, two observers independently rated 175 and 82 droplets, respectively, assigning them to one of five categories. The percentages falling into each category for the two raters were as follows: for contact angle 180° = 81 and 30%; 135° = 14 and 39%; 90° = 4 and 22%; 45° = 0 and 7%; <45° = 0 and 1%. The agreement between the two raters was poor when deciding whether a droplet was closer to 180° or 135° contact angle, but the raters put 96 and 69% of the droplets into the combined category. Also, the droplets rated as having contact angles <45° can be discounted because these can be distinguished by visual cues when viewed from above and are not included in our optical measures. Eliminating those droplets gives the following: >135° = 96 and 76%; 90–135° = 4 and 24%. Using the worst estimates from rater 2, and making the worst case assignment that 76% of droplets have contact angles = 135° and 24% have contact angles = 90°, our secretion rates should be reduced by a correction factor of close to −10%, both because of the distribution of contact angles and because many contact angles are underestimated because of the uneven surface of the epithelium. Many ducts exit in shallow pits, so that when viewed from the side, fully spherical droplets appear to have a contact angle <180°. However, although these arguments suggest that our average rates are accurate, individual rates for single glands will be strongly affected by these factors (see below).

To further validate our spherical approximations of bubble volumes and to ensure that no fluid was partitioning from the small droplets in the oil, control experiments were carried out in which bubbles of water or buffer with different known volumes was injected in oil using silanized, constant-bore microcapillaries (Drummond Scientific) and then followed over time under normal experimental conditions. The difference between the volume of a fluid bubble measured in a microcapillary and the digitally calculated spherical values was within ±10% (n = 11). Side visualization of the injected bubbles with a prism revealed no significant vertical distortion. The x- and y-axes were within ±5%, which is within our measurement error. Volumes were observed over a period of 3 h in bubbles of buffer (Fig. 1D) or water (data not shown). The smallest bubble of buffer (~33 nl) retained its original volume for at least 5.5 h (data not shown). As an additional control for possible optical distortion of images caused by the oil coating, a 0.5-mm calibration grid was covered with oil, and the images obtained were compared with the same grid in air. No size differences were observed between the two conditions.

For HCO\(_3\)-free experiments, all HCO\(_3\) in the Krebs buffer was replaced either with 25 mM HEPES or 1 mM HEPES plus 24 mM NaCl that had been pregassed with humidified 100% \( \text{O}_2 \). The 1 and 25 mM levels of HEPES both maintained the bath \( \text{pH} \) at 7.4 after gassing with \( \text{O}_2 \). The 25 mM HEPES solution provided the best control for intracellular \( \text{pH} \), whereas the 1 mM HEPES solution minimized the chance that alterations in secretion were secondary to ion gradients established between the bath and gland lumen.

Water-saturated mineral oil was prepared by sonicating (~5 min) a mixture of mineral oil and water (50:50 by vol) and was stored at 4°C. Before each experiment, water-saturated oil was vortexed and briefly centrifuged at room temperature. All pharmacological agents were diluted to a final concentration with prewarmed, appropriately gassed bath solution. Carbachol, phenylephrine, isoproterenol, phentolamine, and propranolol were dissolved in deionized water at otherwise indicated and were maintained as stock concentrations. No changes in secretion rate were associated with bath changes.

Reagents. All compounds were obtained from Sigma unless otherwise indicated and were maintained as stock concentrations. Carbachol, phenylephrine, isoproterenol, phentolamine, and propranolol were dissolved in deionized water at a stock concentration of 10 mM. Other stock solutions were as follows: indomethacin, 10 mM in ethanol; acetazolamide, 1 M, and atropine, 10 mM, in DMSO; bumetanide, 0.1 M in an alkaline solution; and TTX, 0.1 mM in 0.2% acetic acid.

Statistics. Data are means ± SE. Student’s t-test for paired or unpaired data or the Mann-Whitney U-test was used as appropriate to compare the means of different treatment groups. The difference between the two means was considered to be significant at \( P < 0.05 \).
RESULTS

Our results are based on sampling 340 single glands in 54 experiments from 12 sheep, with most data obtained from 5 sheep. We monitored 6.3 ± 0.3 glands (range 2–13)/experiment.

Basal secretion. Basal secretion (Fig. 2, A and B) was quantified 1–9 h postharvest for 123 glands from 7 sheep (Fig. 3). No differences in average secretion rates were observed over this 9-h time period. In addition, there was no consistent increase or decrease in basal secretion rates as a function of time in the experimental setup. The mean basal secretion rate for all 123 glands averaged over a 20-min period was 0.57 ± 0.04 nl·min⁻¹·gland⁻¹. Variation in secretion rates among individual glands constituted the largest source of variation in our experiments. Extreme variation could occur within a single tissue preparation. For example, within a contiguous 6.25-mm² area of tracheal tissue from a single sheep exposed to identical treatment, the fastest gland secretion rate was 25 times the slowest rate, i.e., 0.08 and 1.98 nl·min⁻¹·gland⁻¹ in a 1.5-h-old tissue preparation containing 9 glands (Fig. 3). Such intergland differences greatly exceeded differences in average basal secretion rates among sheep, which varied only fourfold, i.e., from a minimum of 0.23 ± 0.08 to a maximum of 0.92 ± 0.21 nl·min⁻¹·gland⁻¹. However, a portion of this wide range of variation may be an artifact of the measurement method. If a larger droplet from a rapidly secreting gland wetted the surface so that its contact angle went from 180° to 135°, its apparent volume would then increase by 11%; if the contact angle was 90°, its apparent rate of increase would be two times its actual rate.

We considered it possible that at least some component of basal secretion might be a response to some combination of trauma from the dissection, mechanical stimulation from the tissue preparation, or temperature fluctuations. However, basal secretion was not inhibited by 1 μM indomethacin or 0.1 μM TTX. Antagonists of cholinergic (10 μM atropine), α-adrenergic (10 μM phentolamine), or β-adrenergic (10 μM propranolol) stimulation, alone or in combination, also did not diminish basal secretion (data not shown). These treatments eliminate several potential types of stimulated secretion but do not rule out the possibility that
what we term basal secretion is actually a response to an unidentified stimulus.

The mean basal secretion rate in each preparation declined over time postharvest. Compared with tissues tested from 1 to 9 h postharvest, basal secretion rates were reduced to ~44% in tissues tested 17–30 h postharvest (0.25 ± 0.03 nl·min⁻¹·gland⁻¹, 168 glands in 8 sheep, \( P < 0.01 \), Mann-Whitney) and fell to 11% in tissues tested 42–56 h postharvest (0.06 ± 0.03 nl·min⁻¹·gland⁻¹, 49 glands in 3 sheep, \( P < 0.01 \) vs. 17–30 h, Mann-Whitney; Fig. 4). These values considerably underestimate the magnitude of the decline in the basal secretory potential of the tissues because they only provide rates for active glands and do not indicate the proportion of glands that had become inactive or had basal secretion rates too low to measure. Cursory observations indicate that the proportion of basally secreting glands also declined over the same time period. We did not quantify that decline because our present method was optimized to provide accurate secretion rates for individual glands by sampling a small area of tissue. This sampling was not done randomly but instead focused on areas of actively secreting glands (see DISCUSSION).

**Secretion stimulated by carbachol.** Gland secretion was markedly increased by the cholinergic agonist carbachol (10 μM; Fig. 2, C and D). The response to carbachol included a short-latency, transient peak followed by sustained secretion that was about one-third of the peak response (Fig. 5). Mean peak secretion rates to carbachol were 15.7 ± 1.2 nl·min⁻¹·gland⁻¹ when measured at 1-min intervals (60 glands in 5 sheep), with peak responses in some glands reaching ~38 nl·min⁻¹·gland⁻¹.

In pig trachea, ACh produces sustained mucus secretion for at least 2 h (37). To establish a basis for correlating secretion of individual glands with sustained secretion of the entire epithelium, we used the average value of sustained secretion after the initial transient. For 10 glands in 5 sheep, sustained secretion to carbachol, defined as the period 5–20 min post-stimulation, was 4.5 ± 0.5 nl·min⁻¹·gland⁻¹ (Fig. 5). As with basal secretion, rates of carbachol-stimulated secretion varied >10-fold among glands, i.e., from 3.1 to 33.4 nl·min⁻¹·gland⁻¹ in one tissue preparation containing seven glands in an area of 6.25 mm². In addition, when secretion rates were tracked at 1-min intervals, we observed marked differences among glands in the temporal patterns of secretion, including oscillations in the secretory rate (Fig. 6). In spite of these large gland-to-gland variations, the mean peak secretory responses to carbachol across sheep varied only 1.5-fold (Fig. 7). In contrast to the decline in basal secretion, the rate of gland secretion stimulated by carbachol was stable for a period of at least 1 day after harvesting (Fig. 8).

**Secretion stimulated by phenylephrine.** The \( \alpha \)-adrenergic agonist phenylephrine (10 μM) stimulated peak gland secretion of only 0.8 ± 0.1 nl·min⁻¹·gland⁻¹ (39 glands, 5 sheep), a value ~5% of the average peak response to carbachol (Fig. 9A, inset). The response to phenylephrine was transient, with secretion rates returning to basal values within 5–10 min after stimulation. Thus, when compared over longer time periods, the response to \( \alpha \)-adrenergic stimulation is trivial compared with cholinergic stimulation.

The small response to phenylephrine was unexpected based on prior reports of strong secretion in the cat to \( \alpha \)-adrenergic agonists (22, 29). To determine if the difference represented a species or methodological difference, we also studied secretion in tracheas from two cats, four pigs, and two humans. In agreement with the prior reports, we observed that phenylephrine stimulated copious gland secretion in the cat of a magnitude similar to the response to carbachol (Table 1). In contrast, pigs and humans responded, like sheep, with large responses to carbachol but with small, transient responses to phenylephrine (Fig. 9B, inset; Fig. 9C; and Table 1).

**Secretion stimulated by isoproterenol.** The \( \beta \)-adrenergic agonist isoproterenol (10 μM) stimulated peak gland secretion 1.8 ± 0.7 nl·min⁻¹·gland⁻¹ (18 glands, 2 sheep), equivalent to ~9% of the average peak response to carbachol. The response to isoproterenol was
transient and returned to baseline within 10–20 min after the treatment (Fig. 10).

**Inhibition of secretion with bumetanide.** In many epithelia, fluid secretion depends on secretion of Cl\(^{-}\) or HCO\(_3\)^-. The Na\(^+\)-K\(^+\)-2Cl\(^{-}\) cotransporter NKCC1 is a common means for elevating intracellular Cl\(^{-}\) concentration, and in many tissues, its inhibition with bumetanide eliminates the major portion of secretion. We found that 100 \(\mu\)M bumetanide had highly variable effects on basal gland secretion, reducing secretion of individual glands by 4–83%. The mean residual basal secretion after bumetanide was 65 ± 19% (25 glands in 3 sheep, \(P = 0.06\), not significant; Fig. 11A). In contrast, for carbachol-stimulated secretion, the inhibitory effect was more effective and consistent, reducing peak carbachol-stimulated secretion to 45 ± 14% of the control value (24 glands from 3 sheep, \(P < 0.01\), Fig. 11B).

**Inhibition of secretion by HCO\(_3\)^- replacement.** HCO\(_3\)^- mediated fluid secretion also plays a role in submucosal gland function (2), but the mechanisms for HCO\(_3\)^- transport are poorly understood, and no specific, reliable inhibitors of the known HCO\(_3\)^- transporters are available. Therefore, we assessed the contribution of HCO\(_3\)^- transport to gland mucus secretion by replacing serosal HCO\(_3\)^- with either 1 or 25 mM HEPES (see METHODS). In response to HCO\(_3\)^- removal (Fig. 11), basal secretion was reduced to 45 ± 14% of control (39 glands from 3 sheep, \(P < 0.01\), and peak carbachol-stimulated secretion was reduced to 33 ± 10% of control (33 glands from 3 sheep, \(P < 0.01\)). Replacement of HCO\(_3\)^- with either 25 mM HEPES or 1 mM HEPES plus 24 mM

![Fig. 6. Variations in secretory responses of single glands to carbachol. Each graph shows the response rates of an individual gland. Responses were selected to show large transients (A and B), small transient (C), and oscillating response (D). Carbachol (10 \(\mu\)M) was present from 20 to 40 min.](image)

![Fig. 7. Mean peak secretory responses to carbachol are similar across sheep. Each bar is average of all glands tested from one sheep. Smallest and largest mean responses differ ~1.5-fold. No. of glands per sheep is shown above bars.](image)

![Fig. 8. Single-gland peak secretion rates to carbachol as a function of time postharvest. Each symbol represents the peak secretion rate of a single gland sampled at 1-min intervals after stimulation with 10 \(\mu\)M carbachol. Data are from 60 glands in 5 sheep (S12–S16); all glands from one sheep have the same symbol. Time scale is compressed after 5-h time point. Note scale break on x-axis.](image)
NaCl produced similar inhibition of carbachol-stimulated secretion, i.e., 60 ± 16% (10 glands in 2 sheep) and 75 ± 21% (16 glands in 2 sheep), respectively.

Inhibition of secretion by bumetanide plus HCO₃⁻ replacement. Variable responsiveness to each of these inhibitors might be expected if gland fluid secretion is mediated by a varying combination of Cl⁻ and HCO₃⁻ transport. However, for basal secretion, joint treatment with both bumetanide plus HCO₃⁻ replacement reduced secretion to 42 ± 15% of control (35 glands in 2 sheep, P < 0.01, Fig. 11), which did not differ significantly from the inhibition produced by HCO₃⁻ replacement alone (P > 0.8). In contrast, for carbachol-stimulated secretion, the joint treatment almost abolished secretion, leaving residual secretion of only 8 ± 2% of the control value (30 glands from 2 sheep, P < 0.01, Fig. 11). Although greatly reduced in amount, residual secretion was still significantly greater than basal secretion (P < 0.05), suggesting the existence of at least one additional mechanism for mucus secretion.

DISCUSSION

Advantages of the single gland optical method. When studying differences in the amount or composition of airway secretions caused by different agonists, species, or region of airway or disease state, single-gland studies can distinguish factors that are confounded in pooled samples. Such factors include differential recruitment or loss of distinct populations of glands, changes in gland number or gland size, or temporal secretion properties.

Prior studies of secretion rates by individual airway glands are relatively rare (Table 1). In an early study of individual gland secretions, powdered tantalum was placed on the airway surface to reveal hillocks of mucus that formed above gland ducts that were subsequently visualized with neutral red staining (27). This method is typically used to count secreting glands (41), but, by making assumptions about the shape of the hillocks, it can also be used to quantify secretion for individual glands (7, 13–15, 28). For more accurate quantification of single gland secretion, Quinton (29) developed the oil-coating method and then collected secretions from excised tissues at timed intervals with constant-bore micropipettes. Ueki and colleagues (39) developed a similar micropipette method for collecting secretions from individual glands in situ without using oil. They measured single-gland secretion induced by autonomic stimulation (39), mechanical stimulation of the larynx (11), gastric irritation (10), and various autonomic mediators (22). In more recent studies, single-gland secretion in pig bronchi was studied with video microscopy with a water-immersion lens (18). Secretion rates were not quantified, but the latency of the rapid response to carbachol was estimated by dilation of the gland duct and emergence of particles from the gland. Finally, methods to study isolated glands have been developed (30, 34) and used to quantify mucus secretion in the cat with a variety of markers, such as glycoconjugates and Na⁺ efflux (see Ref. 33 and references therein).

The discontinuation of the micropipette method is understandable, given the technical difficulties involved. Its restriction to cats probably arises from the relative ease with which cat mucus can be collected. In our studies, we find cat mucus to be less viscous than the mucus of the other species studied (unpublished data). The collection and manipulation of tiny quantities of airway mucus from other species presents technical challenges that are circumvented by optical methods.

Compared with micropipette collections, the optical method allows secretion rates to be quantified more frequently and in more glands. The average gland
number per experiment was only six glands in the present work because we sampled a small area (6.25 mm²) to allow greater resolution. Rapid increases in the cost effectiveness of digital imaging will make this trade-off unnecessary. The relative accuracy of pipette and optical methods was not specifically compared, as each would appear to depend mainly on details of execution. Therefore, it is interesting that they yield very similar estimates of rates (Table 1) even before applying any corrections to our data for surface wetting (see METHODS). This could be a fortuitous result of offsetting errors. In the micropipette method, small gland openings and slowly secreting glands are probably underrepresented in the sample. In the optical method, these slowly secreting glands are included, whereas some of the fastest secreting glands are excluded because of merging, which would tend to reduce the average rate, but the volume of droplets that partially adhere to the surface is slightly overestimated. We are further developing our method to decrease this source of error.

In micropipette studies, the composition and physical properties of single-gland mucus can be studied using microanalytical techniques (22, 29). These can be highly accurate, but they require great skill and are labor intensive. The optical methods we have developed can easily be adapted to measure physical and chemical properties of individual gland secretions. In

![Figure 10](image)

**Fig. 10.** Isoproterenol-stimulated gland secretion. Representative secretion rates of isoproterenol (10 μM)-stimulated gland secretion from a sheep are shown. Each closed circle denotes average basal and stimulated secretion rates of 5 glands.

![Figure 11](image)

**Fig. 11.** HCO₃⁻ and Cl⁻ involvement in gland secretions. A: inhibition (%) in the basal secretion rate in the presence of 0.1 mM bumetanide (Bm) and/or HCO₃⁻ replacement (HEPES). Data are means ± SE from 25–39 glands and 4–7 separate tissue preparations from 3–4 sheep. ♠Significantly different from control, *P < 0.01.

B: inhibition (%) in the carbachol-stimulated secretion rate by bumetanide and HEPES. Data are means ± SE from 24–33 glands and 4–5 separate tissue preparations from 3–4 sheep. **Significantly different from control, P < 0.01.

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**Table 1. Secretion rates of single submucosal glands**

<table>
<thead>
<tr>
<th>Study</th>
<th>No. of Glands</th>
<th>No. of Animals</th>
<th>Basal Secretion, nl·min⁻¹·gland⁻¹</th>
<th>Stimulated Secretion, nl·min⁻¹·gland⁻¹</th>
<th>Type of Stimulation</th>
<th>Reference No.</th>
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<td>Quinton</td>
<td>~35</td>
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<td>~9–20</td>
<td>Bethanechol</td>
<td>29</td>
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<td>Ueki et al.</td>
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<td>16 Cats</td>
<td>9.1 ± 0.6</td>
<td>16.4 ± 0.8</td>
<td>Methoxamine</td>
<td>39</td>
</tr>
<tr>
<td>German et al.</td>
<td>14?</td>
<td>5 Cats</td>
<td>~10–18.6</td>
<td>47.8 ± 4.0</td>
<td>Touch laryngeal</td>
<td>11</td>
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<tr>
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<td>7.9 ± 0.7</td>
<td>17.4 ± 1.7</td>
<td>Gastric irritation</td>
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<td>20.9 ± 1.9</td>
<td>ACh</td>
<td>22</td>
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<td>12.8 ± 0.6</td>
<td>Isoproterol</td>
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<td>15.7 ± 1.2</td>
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<td>Phenylephrine</td>
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<td></td>
<td>25</td>
<td>2 Cats</td>
<td>0.9 ± 0.7</td>
<td>11.8 ± 0.6</td>
<td>Phenylephrine</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2 Humans</td>
<td>1.8 ± 0.1</td>
<td>1.2 ± 1.0</td>
<td>Phenylephrine</td>
<td>11</td>
</tr>
</tbody>
</table>

Data are means ± SE. ?, Unknown.
experiments now underway in collaboration with Drs. Alan Verkman and S. Jayaraman, Na\(^+\), Cl\(^-\), and pH are quantified in situ by injecting ratiometric fluorescent indicators in the undisturbed bubbles of mucus as they are secreted under oil from the gland duct. Viscosity is measured using fluorescence recovery after photobleaching.

Limitations of the method. This method is not optimal for long-term monitoring of secretion because the accumulating mucus bubbles fuse or lose their spherical shape. We achieve long-term monitoring by periodically collecting the secretions, but the collections introduce gaps in the monitoring and are labor intensive. Thus this method complements the method of Ballard et al. (2) and Trout et al. (37, 38) in which bulk mucus is collected for several hours from the entire bronchi. The optical method also eliminates the natural interaction between gland secretions and the surface epithelium. For our present purposes, that simplification is useful, but it is also important to determine how the transport properties of surface epithelium and glands interact to determine the depth and composition of airway surface liquid, which in turn affects mucociliary clearance (40, 41).

Potential artifacts of the optical method arise mainly from the treatment required to obtain good optical images. Optimal imaging requires a flat, dry surface, and this requires the mucosa to be dissected free of cartilage, stretched, cleaned, dried, and oiled. These treatments might influence gland secretion, although no obvious differences were observed when comparing gland secretions from tracheal samples that had intact cartilage and were only lightly blotted. However, it is possible that surface drying and oil coating might block some gland duct openings and prevent or slow secretion unless strongly stimulated. That could contribute to the wide variation observed in basal secretion, including the lack of such secretion in some glands that did respond to stimulation.

Basal secretion. Basal gland secretion in situ is \(>10\)-fold greater than secretion of isolated mucosa (Table 1), indicating the importance of parasympathetic tone. About one-half of the basal secretion in isolated mucosa was resistant to combined treatment with bumetanide and HCO\(_3\)\(^-\) replacement. Basal and carbachol-stimulated secretion differed in several respects. Basal secretion was much smaller and more variable (in relative terms) than carbachol-stimulated secretion, it was less affected by inhibitors, and it diminished more rapidly as a function of time postharvest. Because basal secretion was lost in many glands that remained fully capable of secreting to carbachol, both the decline and variability may be secondary to changes in the numerous local mediators that affect gland secretion and are presumably released during dissection and manipulation. Because gland secretion in vivo is controlled by a host of powerful neural and humoral factors, the physiological significance of the residual basal secretion observed in these isolated preparations is uncertain.

Evidence for subpopulations of glands. In prior studies of individual gland secretion, a wide variation in secretion rates was noted (29, 39) and that was again observed in our studies. However, whereas gland secretion rates in single cats were reported to vary 2- to 3-fold (39), we documented \(>12\)-fold differences in phenylephrine-stimulated secretion, i.e., 2.9 and 36.6 nl/min\(^{-1}\)/gland\(^{-1}\) within one small patch of a cat tracheal preparation containing 10 glands. Basal and carbachol-stimulated gland secretion in sheep trachea showed similar wide variations in secretion rates by individual glands. Moreover, gland secretion rates did not form a normal distribution, suggesting that discrete gland populations exist. Previous studies noted three different types of gland morphology (18) and detected marked differences among glands in the expression of the glycoprotein gene MUC7 (31). It will be important to determine if any of these features are correlated, if glands show diversity in other features, and if any of these features have functional consequences. If distinct subpopulations of glands can be identified, it is possible that they will be differentially affected by airway diseases.

Complex responses to carbachol. On average, carbachol produced a transient peak in gland secretion that was \(>28\)-fold greater than basal secretion (\(<9\)-h-old tissue preparations), followed by sustained secretion of approximately one-third of the peak rate, with the same wide variation in actual rates observed for basal secretion. When examined individually, different glands showed distinct temporal response patterns, with prominent oscillations of rate in some glands (Fig. 6D). Variations in rate might arise from several sources. They could have a trivial basis. For instance, Inglis et al. (18) observed a transient block of secretion by a particle that occluded a duct. They could be oscillations in myoepithelial cell tension, although none were reported in direct measures of tension of isolated cat and dog glands (34). Finally, the oscillations, which had periods of 2–3 min, might reflect oscillations of fluid secretion secondary to oscillations of intracellular Ca\(^{2+}\) concentration. Oscillations with similar frequency occur in Calu-3 cell monolayers when stimulated with isoproterenol or thapsigargin (26, 32).

There is disagreement in the literature about whether responses to carbachol are transient or sustained. In cat trachea (29) and pig distal bronchi (37), mucus secretion was sustained (for at least 2 h in pig), whereas, in bovine trachea, secretion returned to baseline within \(~5\) min (41). It is not yet known if these are species or methodological differences.

Responses to carbachol in the sheep were robust. We observed undiminished responses to carbachol in glands that had been isolated for \(>48\) h previously. This is consistent with evidence that contractions of tracheal submucosal glands to cholineric agonists were undiminished up to 3 days postharvest (34).

Ineffectiveness of adrenergic stimulation. Neither \(\alpha\)-nor \(\beta\)-adrenergic stimulation was an effective agonist for mucus secretion in sheep. A small response to the \(\beta\)-adrenergic agonist isoproterenol is consistent with
prior reports for cats (22, 29). In contrast, the ineffec-
tiveness of the α-adrenergic agonist phentolamine was
unexpected because in cats phentolamine is similar in
effectiveness to cholinergic stimulation (29, 39). We
confirmed a large response to phentolamine in cats but
went on to show that it is ineffective in sheep, pigs, and
humans. The basis for these species differences is
presently unknown.

Role of NKCC-mediated (bumetanide-inhibitable)
Cl\(^{-}\) secretion. The inhibitory effect of bumetanide on
gland mucus secretion has been studied in four species
with very different results. Bumetanide inhibition of
cholinergically stimulated secretion was 55% in sheep
(this paper), 70% in pigs (37), and 85% in cows (41). In
contrast, phentolamine-stimulated gland secretion in
cats was unaffected by bumetanide (6). Corrales et al.
(6) interpreted the insensitivity to bumetanide (and to
anion substitutions) to mean that gland fluid secretion
was passively produced after the release of osmotically
active components of secretory granules. However,
phentolamine-mediated secretions in cats are less vis-
cous than cholinergically mediated secretions (22),
and, in pigs, inhibition of fluid secretion with bumet-
ane and dimethylamiloride caused scantier, thick-
ened secretions (38). On the basis of these results, and
additional arguments that follow (see below), we sug-
gest that gland secretion may rely on an as yet unspec-
ified way on HCO\(_3\)-mediated fluid secretion and that
this reliance varies across species.

Role of HCO\(_3\) in gland secretion. Replacement of
HCO\(_3\) with HEPES and the simultaneous change of
gassing from a 95% O\(_2\)-5% CO\(_2\) mixture to air reduced
basal secretion by 55% and cholinergically stimulated
peak secretion by 66%. The basis for this profound
inhibition of secretion is unknown. Basal short-circuit
current in Calu-3 cells, which is thought to be predom-
inantly HCO\(_3\)-dependent (8, 21), is also reduced by
>70% by replacement of HCO\(_3\) with HEPES (35). How-
ever, it is unlikely that secreted gland mucus contains
high levels of HCO\(_3\) based on direct ion mea-
surements of uncontaminated mucus from cat submu-
cosal glands, which suggest that HCO\(_3\) levels in mucus
are similar to bath values (29), and based on acidic pH
measurements of airway surface liquid in the ferret
after carbachol stimulation (20). One possibility is that
the fluid secreted by acinar serous cells is indeed rich
in HCO\(_3\), but most of the HCO\(_3\) is absorbed before the
final mucus is secreted. This and other possibilities
need to be investigated.

In conclusion, we describe an optical method for
quantifying single-gland secretion rates with improved
temporal resolution. The method should be applicable
to most gland-containing epithelia. We introduced the
method by focusing on secretion rates, but, because
secretions from each gland are isolated and protected
from evaporation, it is feasible to inject indicators for
optical monitoring of pH, ionic content, and viscosity.
Gland ducts can subsequently be injected with mark-
ers, followed by fixation and sectioning, to relate gland
structure and function. Single-gland methods are re-
quired if glands are heterogeneous, as our initial re-
results suggest. When used to compare glands from nor-
mal and diseased tissues, these methods will allow the
testing of hypotheses about the contributions of gland
dysfunction to the pathogenesis of airway diseases.

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REFERENCES

1. Ballard ST, Fountain JD, Inglis SK, Corboz MR, and Tay-
lor AE. Chloride secretion across distal airway epithelium: re-
lationship to submucosal gland distribution. Am J Physiol Lung

2. Ballard ST, Trout L, Bebok Z, Sorscher EJ, and Crews A.
CFTR involvement in chloride, bicarbonate, and liquid secretion
by airway submucosal glands. Am J Physiol Lung Cell Mol

3. Basbaum CB, Jany B, and Finkbeiner WE. The serous cell.

cloned by nuclear transfer from a cultured cell line. Nature 390:
64–66, 1996.

5. Choi HK, Finkbeiner WE, and Widdicombe JH. A compar-
sive study of mammalian tracheal mucus glands. J Anat 197:

6. Corrales RJ, Nadel JA, and Widdicombe JH. Source of the
fluid component of secretions from tracheal submucosal glands

7. Davis B and Nadel JA. New methods used to investigate the
control of mucus secretion and ion transport in airways. Environ

8. Devor DC, Singh AK, Lambert LC, DeLuca A, Frizzell RA,
and Bridges RJ. Bicarbonate and chloride secretion in Calu-3
human airway epithelial cells. J Gen Physiol 113: 743–760,
1999.

9. Engelhardt JF, Yankaskas JR, Ernst SA, Yang Y, Marino
CR, Boucher RC, Cohn JA, and Wilson JM. Submucosal
glands are the predominant site of CFTR expression in the

10. German VF, Corrales R, Ueki IF, and Nadel JA. Reflex
stimulation of tracheal mucus gland secretion by gastric irrita-

11. German VF, Ueki IF, and Nadel JA. Micropipette measure-
ment of airway submucosal gland secretion: laryngeal reflex. Am

CFTR in Calu-3 human airway cells: channel properties and role in

13. Haxhiu MA, Cherniack NS, and Strohl KP. Reflex responses
of laryngeal and pharyngeal submucosal glands in dogs. J Appl

MA. Pathways and mechanisms involved in neural control of
laryngeal submucosal gland secretion. J Appl Physiol 75: 2347–
2352, 1993.

15. Hejal R, Strohl KP, Erokwu B, Cherniack NS, and Haxhiu
MA. Effect of hypoxia on reflex responses of tracheal submu-

16. Inglis SK, Corboz MR, and Ballard ST. Effect of anion secre-
tion inhibitors on mucin content of airway submucosal gland
ducts. Am J Physiol Lung Cell Mol Physiol 274: L762–L766,
1998.

17. Inglis SK, Corboz MR, Taylor AE, and Ballard ST. Effect of
anion transport inhibition on mucus secretion by airway submu-

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MUCUS SECRETION FROM SHEEP SUBMUCOSAL GLANDS


