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Acinar origin of CFTR-dependent airway submucosal gland fluid secretion

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Wu JV, Krouse ME, Wine JJ. Acinar origin of CFTR-dependent airway submucosal gland fluid secretion. *Am J Physiol Lung Cell Mol Physiol* 292: L304–L311, 2007. First published September 22, 2006; doi:10.1152/ajplung.00286.2006.—Cystic fibrosis (CF) airway disease arises from defective innate defenses, especially defective mucus clearance of microorganisms. Airway submucosal glands secrete most airway mucus, and CF airway glands do not secrete in response to VIP or forskolin. CFTR, the protein that is defective in CF, is expressed in glands, but immunocytochemistry finds the highest expression of CFTR in either the ciliated ducts or in the acini, depending on the antibodies used. CFTR is absolutely required for forskolin-mediated gland secretion; we used this finding to localize the origin of forskolin-stimulated, CFTR-dependent gland fluid secretion. We tested the hypothesis that secretion to forskolin might originate from the gland duct rather than or in addition to the acini. We ligated gland ducts at various points, stimulated the glands with forskolin, and monitored the regions of the glands that swelled. The results supported an acinar rather than ductal origin of secretion. We tracked particles in the mucus using Nomarski time-lapse imaging; particles originated in the acini and traveled toward the duct orifice. Estimated bulk flow accelerated in the acini and mucus tubules, consistent with fluid secretion in those regions, but was constant in the unbranched duct, consistent with a lack of fluid secretion or absorption by the ductal epithelium. We conclude that CFTR-dependent gland fluid secretion originates in the serous acini. The failure to observe either secretion or absorption from the CFTR and epithelial Na^+ channel (ENaC)-rich ciliated ducts is unexplained, but may indicate that this epithelium alters the composition rather than the volume of gland mucus.

innate defense; mucus

HUMAN AIRWAYS ARE NORMALLY kept sterile by potent innate defense mechanisms, including mucus clearance (15), but most cystic fibrosis (CF) patients are killed by chronic bacterial infections of the airways. Remarkably, infecting organisms are confined to the airway mucus until very late in the disease (34). Submucosal glands produce most of the airway mucus (22, 30), gland secretions protect against infections (4), and secretion by CF airway glands is defective (9–12, 23, 26, 29, 32). In particular, mucus secretion in response to VIP or forskolin is absent in CF airway glands but not in glands from many other airway diseases (11). This result indicates that CFTR, a cAMP-activated, apical anion channel that is defective or missing in CF patients, is essential for cAMP-mediated gland secretion. However, the precise role of CFTR in gland secretion has not been established, and even the location of CFTR within the glands is controversial.

Based on serial reconstructions of human glands (19), CFTR immunohistochemistry, and direct observation with Nomarski differential interference contrast (DIC) microscopy, a four-compartment model has been developed for airway gland secretion (Fig. 1*A*) (13, 32). In this model, a gland comprises distal serous acini and mucous cell tubules, which all empty into a large diameter collecting duct that connects to the surface via an unbranched ciliated duct (19). According to a report based on a potent and well-characterized polyclonal antibody, CFTR is primarily located in serous acini (5); however, more recent work using sensitive monoclonal antibodies found abundant CFTR in the ciliated ducts but saw little or no CFTR signal in the serous acini (17).

Fluid secretion in response to VIP/forskolin is absent in CF glands and is therefore dependent on functional CFTR (11). In an attempt to reconcile this functional evidence with the evidence that CFTR is more heavily expressed in ciliated duct cells (17), we considered two hypotheses. First, we reasoned that in CF glands, VIP/forskolin might stimulate fluid secretion from the acini via a non-CFTR-dependent mechanism, but the mucus might then be blocked by hyperabsorption in the ducts. We reasoned that ductal hyperabsorption and blockage could occur if the lack of functional CFTR in the CF ducts caused the epithelial Na^+ channel (ENaC) in the duct to be hyperactive, as it does in airway surface cells (6, 28). This latter possibility was recently rendered unlikely by experiments in which ENaC blockers were applied to normal and CF glands without increasing secretion in normal glands or rescuing secretion in CF glands (10).

We next hypothesized that VIP/forskolin-stimulated secretion might actually originate from the CFTR-rich ciliated duct, just as pancreatic fluid secretion is known to be primarily of ductal origin (27); the studies reported here test that hypothesis. We used two different methods to determine if ciliated ducts either secrete (or absorb) significant amounts of fluid. We ligated the ducts at one or both ends and compared fluid transport across the ductal epithelium with that in the other compartments. We also directly monitored mucus flow rates within different compartments of the gland using time-lapse, DIC (Nomarski) microscopy, and particle tracking.

METHODS

Tissues and buffer. Human tracheal trimmings were obtained as surgical scrap from donor lungs used for transplants. Pig tracheas were obtained from fresh carcasses following acute experiments carried out for other purposes; no pigs were killed specifically for the present experiments. In one case a bronchus was obtained after informed consent from a subject who was transplanted for interstitial lung disease. Pig or human tissues were maintained until use in cold Krebs-Ringer bicarbonate buffer gassed with 95% O_2 and 5% CO_2. The Krebs-Ringer bicarbonate buffer composition was 115 mM NaCl, 2.4 mM K_2HPO_4, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM glucose, and 1.0 μM indomethacin. The pH was 7.4, and osmolarity was adjusted to ~290 mosM. Pieces

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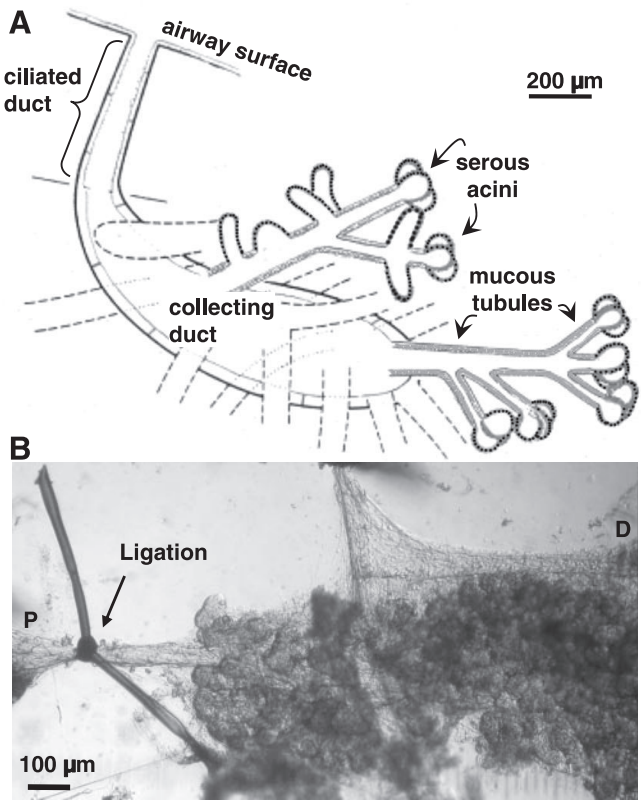

Fig. 1. *A:* 4-compartment model of airway submucosal gland. A reconstruction from Meyrick and Reid based on serial sections of a human airway bronchial gland with each compartment indicated. Only 2 of multiple branches were reconstructed, and the rest are shown as dashed lines. At issue are the compartmental locations of CFTR and the origin of VIP and forskolin-stimulated fluid secretion. *B:* ductal ligation of an isolated gland. A digital micrograph of an isolated pig gland in which the proximal end of the duct near its orifice (P) was ligated with a nylon thread. D, distal (acinar) region of the gland.

of airway ~2 cm^2 were pinned mucosal-side-up, and the mucosa with underlying glands was dissected from the cartilage and pinned on a Sylgard-lined petri dish with the serosa faced up. Muscle fibers and connective tissues were dissected away from a set of submucosal glands, which were then either isolated with ducts intact (i.e., a small piece of airway surface was included with the duct orifice) or left in situ with ciliated duct still attached to the surface epithelium. Ciliated ducts are here defined as the unbranched portion of the duct that runs from the first branch point in the acinar rich area of the gland to the airway surface.

Gland isolation, duct ligation, and optical monitoring of duct diameters. Submucosal glands were isolated so that the entire ciliated duct was accessible, and were then observed with a Wild stereoscopic microscope at ×25 magnification with lateral illumination via a flexible, fiber-optic illuminator (model 190; Dolan-Jenner Industries, Lawrence, MA). Gland ducts were ligated with 22-μm nylon threads using either a single ligation near the orifice (Fig. 1*B*) or a second ligation between the ciliated and collecting duct. Ligated glands were pinned to a Sylgard-lined chamber of a glass slide, and gland duct diameters were monitored by digital imaging with a compound microscope using ×2.5 or ×4 objectives. The initial digital micrograph was made at 22°C and served as the control image against which all subsequent measures were compared. We tested responses to three conditions, each maintained for 20 min: *1*) warming to 37°C in Krebs alone, *2*) addition of 10 μM forskolin, or *3*) with 10 μM carbachol. All measurements were made after ~20 min, all were in the presence of 95% O_2 and 5% CO_2, and all but the first were at 37°C.

In situ gland ligation and optical monitoring of single gland secretion. Glands were exposed as described above, but instead of isolating the glands they were left with ciliated duct still attached to the surface epithelium. Selected glands (up to 13 per tissue) were then ligated at the collecting/ciliated duct junction, leaving a larger number of adjacent, unligated glands to serve as controls. The tracheal mucosa was then prepared for optical monitoring as described previously (14). In brief, the tissue with attached glands was pinned mucosal-side-up in a Sylgard-lined plastic dish with the serosa in the bath (~2 ml vol) and the mucosa in air. The tissue surface was cleaned, dried, and covered with a layer of water-saturated mineral oil; bubbles of mucus that formed at the orifices of the gland ducts in the oil layer were visualized by transillumination with an array of 10-mm diameter, ultra-bright, white-light-emitting diodes, which also revealed the glands and ligatures. Digital images were captured with a Nikon digital camera mounted on a Wild dissecting microscope. For stimulation, the tissue was warmed to 37°C at a rate of ~1.5°C per minute while being continuously superfused with warmed, humidified 95% O_2 and 5% CO_2. Pharmacological agents were diluted to their final concentrations with warmed, gassed bath solution and added to the serosal side by complete bath replacement. The tissue was stimulated with 10 μM forskolin for 20 min followed by 10 μM carbachol unless indicated otherwise.

Monitoring fluid flow within specific compartments of single glands with DIC. Glands were isolated as described above and then further microdissected to prepare relatively clean but intact glands for optimal imaging. When judged suitable for imaging, the glands were transferred to microperfusion chambers on the stage of an upright Nikon Eclipse E600FN Series Microscope equipped with DIC and epifluorescence. They were continuously perfused with Krebs or Krebs-forskolin gassed with 95% O_2 and 5% CO_2 via a pressurized, eight-chamber, solenoid-actuated perfusion system (Automate, San Francisco, CA); temperature was maintained at 35–37°C using a TS-4 Peltier effect temperature controller that warmed the inflow tubing and chamber.

For digital imaging, the microscope is fitted with a Retiga-1300, cooled, 12-bit, color-Bayer Mosaic CCD camera with RGB Liquid Crystal Color Filter Module. The camera is interfaced with a high performance computer running Compix Image capture and analysis software.

For overview images of the gland, we used a ×4 objective. For detailed studies of mucus flow, glands were imaged with a ×40 water-immersion lens (numerical aperture 0.8, 2-mm working distance), and time-lapse digital imaging was used to monitor changes in mucus flow with frame rates of 1 frame/s to 2 frame/min. At this magnification, the field and focal plane are sufficiently restricted so that it is usually necessary to pick a single duct, tubule, or acinus for optical imaging.

Particle movements were first described during in situ studies of single airway glands by Ballard and colleagues (7). We assume as a first approximation that the net longitudinal movement of particles observed within the mucus can be used to measure the bulk flow of the mucus, and that, together with the duct or tubule diameter, the secretion rate can be calculated and expressed as picoliter per minute; if the measurement occurs in the final unbranched duct of the gland, it can be expressed as picoliter per minute per gland. We assume that particles will *1*) accelerate if the surrounding epithelium is secreting, *2*) decelerate if the surrounding epithelium is absorbing, and *3*) be constant if the surrounding epithelium shows no net secretion or absorption.

Reagents. Compounds (Sigma) were made fresh or maintained at −20°C in the following solvents: carbachol and VIP in distilled water, indomethacin in ethanol, and forskolin in Me_2SO. All were diluted 1:1,000 with bath solution (except indomethacin, which was diluted 1:10,000) immediately before use at the concentrations indicated.

Statistics. Data are means ± SE, and Student's *t*-test for unpaired data was used to compare the means of different treatment groups unless otherwise indicated. The difference between the two means was considered to be significant when $P < 0.05$.

RESULTS

Glands ligated near the gland duct orifice show volume expansion when stimulated. Mucus secreted by submucosal glands empties onto the airway surface via the gland duct orifice. We stopped the outflow by ligation to determine if the gland epithelium would continue to secrete mucus in spite of the blockage. If it did, we expected to see visible distension of the gland, as is known to occur in other examples of occluded glandular structures (1). We also needed to know if our microdissection methods created artificial leaks in the glands. As illustrated in Fig. 2, ligated ducts accumulated mucus that expanded the ductal compartments distal to the ligation. The accumulation, which was measured here simply as an increase in ductal diameter at two time points spaced 20 min apart, was readily apparent even under "basal" conditions in which the gland was warmed quickly from 22 to 37°C. In the critical experiment, the gland ciliated and collecting duct diameters were further increased by the addition of 10 μM forskolin. The increase to forskolin was relatively small because the measurement was made after only 20 min, and the latency for glands to begin to respond to forskolin can be 5–10 min, at least when measured by fluid secretion onto the surface (11, 13). Duct diameters were increased still further by the addition of 10 μM carbachol.

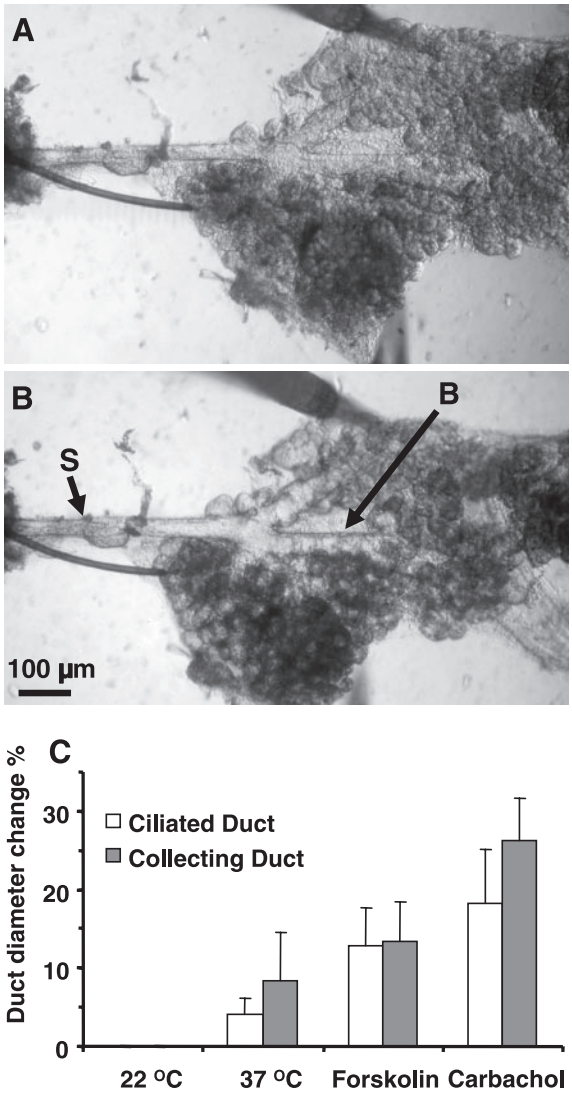

Fig. 2. Swelling of pig glands having a single proximal ligation. *A*: optical micrograph of the gland taken at 22°C. *B*: after stimulation with 10 μM carbachol for 20 min. *C*: summarized duct expansion data ($n = 5$ glands). Arrows: S, straight (ciliated) duct; B, branched (collecting) duct.

The ciliated duct diameter was measured midway between the ligation and the collecting duct, and the collecting duct was measured beyond the first branch point. At room temperature, the average ciliated and collecting duct diameters were 31.1 ± 4.0 and 45.6 ± 3.2 μm, respectively, at baseline conditions ($n = 5$). When warmed to 37°C, the ciliated duct diameter increased by 4.1% to 32.0 ± 3.8 μm, and the collecting duct diameter increased by 8.4% to 48.0 ± 1.8 μm.

Both ciliated and collecting ducts showed larger increases after stimulation. As shown in Fig. 2*C*, the ciliated duct increased by 12.6% to 34.0 ± 3.6 μm when exposed to 10 μM forskolin and by 18.3% to 36.8 ± 5.4 μm after 10 μM carbachol. The corresponding increases in the collecting duct diameter were 13.3% to 48.0 ± 1.8 μm after forskolin and 26.2% to 50.8 ± 2.1 μm after carbachol. All changes were significantly different from the 37°C control ($P < 0.05$) except the ciliated duct response to forskolin ($P = 0.08$). We saw no visible mucus exiting near the ligation site, suggesting little or no leakage resulted from the ligations. Glands were swollen in all compartments but more prominently toward the distal (acinar) regions (see DISCUSSION). In sum, these experiments demonstrate that isolated, ligated glands continue to secrete fluid basally and in response to mediators, that more distal regions show a greater degree of expansion, and that isolated, ligated glands either do not leak or secrete at a rate that overwhelms any leakage.

Double ligations that isolate the ciliated duct demonstrate that secretion originates from distal regions of the gland. To determine if the fluid secreted by ligated glands originates wholly or in part from the ciliated duct, we made dual ligations at both the duct orifice and at the junction between the ciliated and collecting duct (Fig. 3*A*) in a series of glands that were then treated as before with temperature increase and forskolin and carbachol stimulation. Glands with larger ciliated ducts were chosen for these experiments. The average pig ciliated duct diameter under the control condition was 53.2 ± 3.4 μm; the collecting duct was 41.9 ± 11.8 μm. As shown in Fig. 3*B*, the doubly ligated (isolated) ciliated duct diameter showed minimal changes under all conditions, increasing by 0.5 ± 0.7% (0.2 ± 0.4 μm) when warming from 22 to 37°C, by 0.1 ± 1.9% (0.06 ± 0.93 μm) after 10 μM forskolin, and by 1.3 ± 1.5% (0.74 ± 0.79 μm) in 10 μM carbachol ($n = 5$, $P > 0.35$, not significant for all conditions). By contrast, the collecting duct expanded by 5.6 ± 5.6% (0.87 ± 1.6 μm) when warmed, by 16.5 ± 10.9% (4.2 ± 2.3 μm) in forskolin, and by 54.6 ± 8.8% (22.5 ± 6.5 μm) in carbachol ($n = 5$; $P = 0.57$, 0.11, and 0.018, respectively).

Double ligations were also applied to eight submucosal glands isolated from a human donor trachea and a disease control bronchus. The diameters of ciliated ducts did not

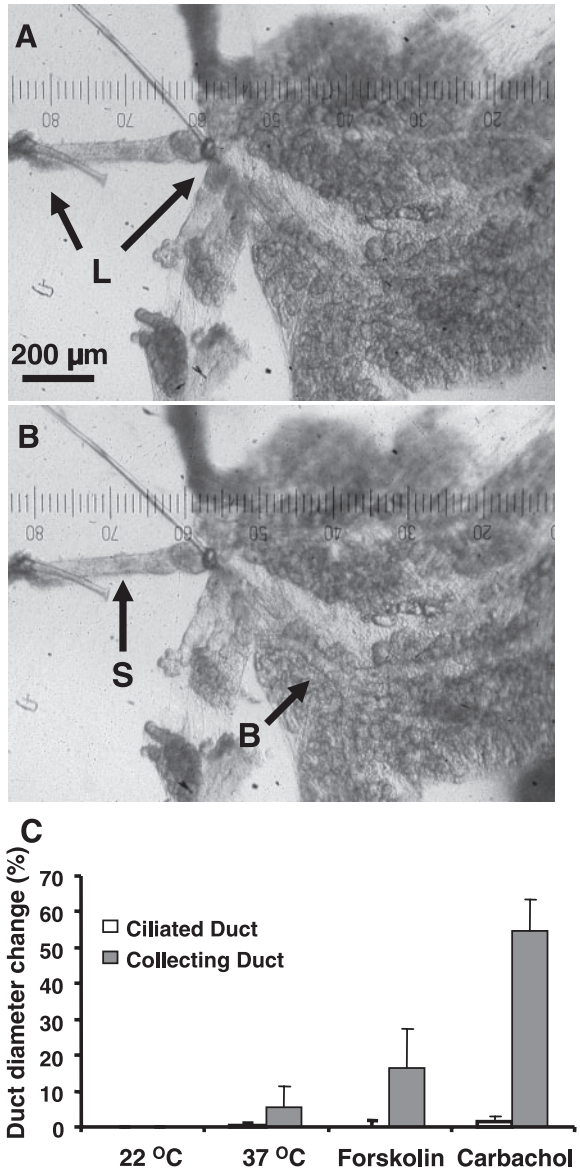

Fig. 3. Swelling of collecting but not ciliated duct after forskolin stimulation of glands with double ligations to isolate the ciliated duct. *A*: optical micrograph of double ligations on an isolated submucosal gland before stimulation. *B:* after forskolin and carbachol stimulation. *C*: summarized data of duct expansion with 2 ligations. Arrows: L, double ligations; S, straight (ciliated) duct; B, branched (collecting) duct.

change significantly for any condition ($P > 0.36$). Extensive connective tissue and the larger size of the human glands hindered the visualization of collecting ducts in all except one tracheal gland from a human donor. In that gland, we measured the collecting duct at five adjacent points where mucus tubules entered the duct. Duct diameter increased by 5.5% after warming (69.4 ± 8.7 μm to 73.2 ± 5.6 μm, $P > 0.3$), by 19.4% (82.9 ± 7.8 μm, $P < 0.004$) after forskolin, and by 35.2% (93.8 ± 7.3 μm, $P < 0.005$) after carbachol.

Single ligations between the ciliated and collecting duct abolished fluid secretion from the open orifice of the ciliated duct. An alternative interpretation of the double ligation experiment is that ductal fluid secretion is stopped by negative feedback mechanisms that detect pressure in the duct. To address that issue, we left the duct orifice open but isolated the duct from the rest of the gland with a single ligation at the distal end of the duct where it originates from the collecting duct. Fluid secretion was then monitored in situ using the optical oil layer method (14). Mucus accumulation under oil at the orifices of ligated glands (isolated ducts) was then compared with neighboring, unligated glands in the same preparation (Fig. 4). We observed no secretion from the ductal openings of 23 ligated glands in experiments from three different pigs, even though the entire ciliated duct was intact in the ligated glands. By contrast, essentially all of the control, unligated glands showed mucous secretion.

To ensure that these results were not due to leakage or general damage to the ligated glands, we flipped the tissue mucosa side down at the end of the stimulation series so that we could visualize the ligated glands. It was then apparent that the ligated glands showed visible swelling throughout from ducts to acini, compared with the adjacent unligated glands, consistent with our observations of isolated glands. We measured at least 15 individual acini in each of three ligated and three unligated glands in a single pig trachea. Assuming a spherical volume for the acini, the mean volume of 45 acini from the ligated glands was 515 ± 5 pl, more than twice the mean acinar volume of 240 ± 2 pl measured in 53 acini from unligated glands. Similar measurements in a single human trachea after carbachol stimulation showed that human gland acini in ligated glands were on average twofold greater in volume (478 ± 13 pl, $n = 16$ acini) than acini from unligated glands (232 ± 2 pl, $n = 53$ acini).

Origins of gland mucus secretion determined by DIC time-lapse imaging. We are pursuing a research program that uses DIC methods to study many aspects of airway gland secretion. As part of that program, we have taken advantage of particles that are observed in mucus (7) to track mucus flow through the various compartments of the airway submucosal glands. As stated in METHODS, particles flowing through a tube that is adding fluid either across the epithelium or via inlet tubes will accelerate, whereas particles in a tube that is doing neither will display a constant rate of flow. Here, we have concentrated on a comparison of particle flow in the acini and in the ciliated duct in response to forskolin stimulation; these are the two gland regions where immunocytochemistry has localized CFTR.

Particles originate in acini and show accelerated movement from the acini. Microdissected but otherwise intact human airway glands from donor trachea were used for these experiments. For all results reported here, the glands had been slowly equilibrated to 37°C and were then stimulated with 5 μM forskolin and allowed to reach steady-state secretion. By visual inspection at low power, particle movement was observed in all regions of the gland, and in all cases the particles moved from the acini toward the ducts (data not shown). When glands were injured, either inadvertently or deliberately, the flow of mucus was altered and could sometimes be seen exiting from the injured region. As will be shown below, this provided an additional method for localizing the origins of secretion. At higher power, particles could be tracked within individual acini (Fig. 5*A*). They always moved toward the proximal portion of the gland, and the calculated secretion rate at their position increased as they moved along the acini (Fig. 5, *A* and *C*). Particles were seen in 14 of 30 acini from 20 human subjects under all experimental conditions, always with the same general trends of distal-to-proximal movement and

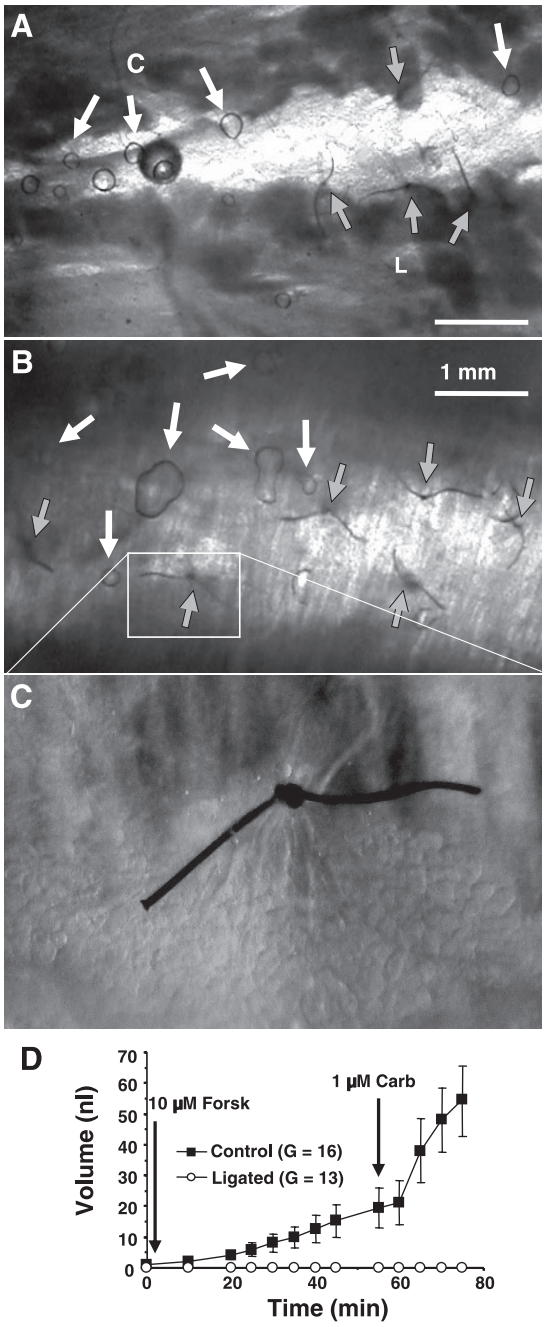

Fig. 4. Absence of ductal secretion after ligation in situ. *A* and *B*: images of airway mucosal surface from pig (*A*) and human (*B*) showing mucus droplets under oil using the method of Joo et al. (14), except transillumination was used. Glands did not secrete fluid after a ligation was made at the junction of the straight duct and the branched collecting duct. Arrows with black outline point to ligated glands, and white arrows point to mucus droplets secreted by adjacent unligated glands. Glands and ligatures are visible through the semitransparent mucosa. *C*: view of a ligated gland in situ from the serosal side. *D*: mucus secretion over time in 16 unligated and 13 ligated glands from a single human trachea. G, glands; Forsk, forskolin; Carb, carbachol.

acceleration, but we observed wide variation in actual particle speeds. Only two acini in steady-state forskolin had particles that gave meaningful data (the particles stayed in focus and were recorded long enough to allow analysis of secretion rates over time).

Particle movement could reverse temporarily upon addition of forskolin. Before the addition of forskolin, particles in the acinus may be either static or moving forward slowly (basal secretion). Shortly after forskolin is added, the acini dilated slightly and particles in the mucus moved backwards transiently before reversing course to flow out of the acinus with accelerating speed (Figs. 5 and 6). We interpret this sequence to represent an initial relaxation of the myoepithelial cells before the onset of forskolin-stimulated fluid secretion.

Particles moved at constant speed within the ciliated duct. In contrast with the acceleration observed as a function of distance in the acini and tubules, particles within the ciliated duct moved at relatively constant speeds along the duct, corresponding to a calculated secretion rate of ~140 pl/min per gland (see METHODS) with virtually no change in secretion rate as a function of distance in micrometers along the duct (0.33 ± 0.25 pl·min^{-1}·μm^{-1}, $n = 7$ subjects; Fig. 7, *A* and *B*), consistent with that region of the gland neither adding nor subtracting volume from the flowing mucus. Particles were analyzed in ducts from seven humans, always with the same general trends of distal-to-proximal movement and constant speed, but we again observed wide variation in actual speeds of movement among different gland ducts.

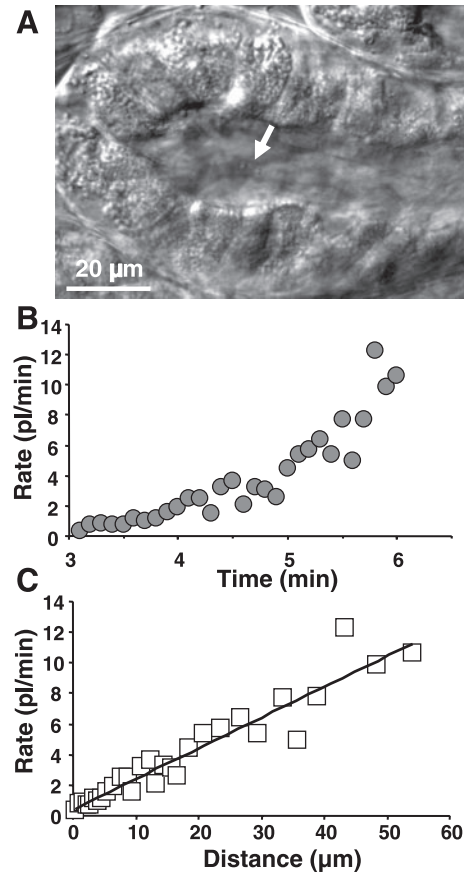

Fig. 5. Particle movement in human gland acini under steady-state forskolin stimulation. *A*: differential interference contrast (DIC, Nomarski) digital micrograph of gland acinus showing 2 particles used for tracking mucus movement. *B*: the calculated secretion rate of the mucus computed at the particle location every 10 s. *C*: the data are replotted to show calculated secretion rate as a function of distance along the acinus. The approximately linear increase in rate is interpreted to reflect that addition of fluid from increasing numbers of cells that contribute to the mucus stream.

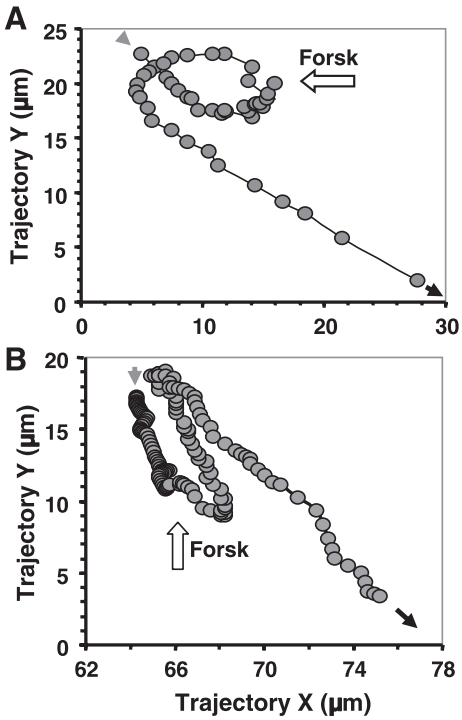

Fig. 6. Particle movement trajectories and rates in human submucosal gland acini after stimulation with forskolin. *A* and *B*: mucus particle positions as a function of time and stimulation in 2 human acini. The duct diameters are 62 and 26 μm, respectively. Both of the particles are at the middle third of the duct, which should preclude any edge effect by the duct. Each symbol represents the *x*,*y* coordinates of a particle at 5-s intervals. Notice the random and slow movements before the addition of forskolin, and then more rapid and directed movements afterward. The *z*-axis was not tracked explicitly, but because particles were chosen that stayed in focus during the period of observation, the motion is primarily in the plane of observation.

DIC measurements of particle movements in a distally ligated duct showed no stimulation of particle movement. In a single experiment (Fig. 7), we measured mucus particle displacement vs. time for a straight duct ligated at the duct-gland border. A comparison with a nonligated duct is shown. Initially, there was basal movement of particles in the ducts equivalent to a secretion rate of 5 pl/min per gland. The origin of this movement is unknown (see DISCUSSION), and it was only about one-tenth of the basal movement in the nonligated duct (70 pl/min per gland) chosen for comparison in Fig. 7. After stimulation with 5 μM forskolin, particle movement in the unligated duct increased 2.7-fold, in contrast with particles in the ligated duct, which showed almost no change in speed (7 pl/min per gland). These observations are not consistent with significant ductal fluid secretion.

Nicked duct experiment shows static mucus between the nick and the duct orifice. Also in a single experiment, a small hole was made approximately halfway along a ciliated duct in an isolated pig gland, and mucus particles were observed at the region of the nick and again at the ductal orifice. As diagrammed in Fig. 8, particles flowed from the distal regions of the gland to the nicked region and then exited through the nick. By contrast, mucus in the duct between the nick and the ductal orifice was static, even though cilia were beating; this condition is not consistent with significant fluid secretion from the distal, ciliated portion of the duct.

DISCUSSION

It is generally accepted that the acini are the origin for fluid secretion in most glands, but the interlobular ducts of the pancreas are an exception (1) and are also, interestingly, the exclusive site of CFTR expression in the pancreas (18). Based on that, and on a recent report of high expression of CFTR in the ciliated ducts but little or none in the acini of airway glands (17), we reinvestigated the premise that the serous acini are the source of fluid secretion in response to forskolin, and that loss of functional CFTR from the serous acini is the reason that CF glands fail to secrete to mediators like forskolin that raise intracellular cAMP concentration (11, 13, 32).

We used duct ligation and direct observation of particle movements to determine if the ducts contribute fluid to the gland mucus. Neither method provided any evidence for ductal secretion in response to forskolin, but instead indicated a more distal source for secretion. Direct observation of acinar particle movements showed that fluid secretion in response to VIP/forskolin originates in the serous acini with no evidence for fluid addition from the duct. Our results are consistent with an ultrastructural study of rat submucosal glands, where the mean number of parallel fibrils in tight junctions between serous cells (3.6 ± 0.4) was significantly smaller than those between mucous tubules (5.1 ± 0.6) or ciliated duct cells (8.5 ± 0.7). Furthermore, colloidal lanthanum permeated the tight junctions between serous cells up to the level of the acinar lumen, but did not penetrate between the mucous or ciliated cell junctions. These results suggest that tight junctions between serous cells are more permeable to small water-soluble solutes, which is consistent with a secretory function (25).

Some details relevant to gland function and the methods used. Glands ligated at the duct orifice showed larger volume expansion in the collecting duct than in the unbranched, ciliated duct. That could indicate that this region is more compliant. It is also possible that the expansion occurs because mucus forms a

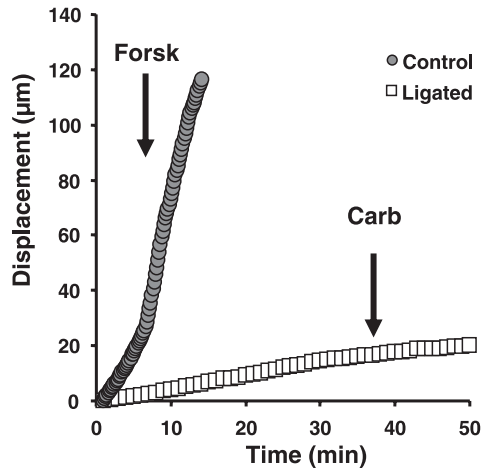

Fig. 7. Comparison of basal and stimulated mucus flows in ligated and unligated ducts. Displacement as a function of time in an unligated control duct (filled circles). Each curve represents an average of 4 particle movements. Particles in the mucus moved at a near constant speed until stimulated with 5 μM forskolin; they then increased ~2.7-fold to a second near-constant speed; subsequent stimulation with carbachol produced an even greater increase (not shown). By contrast, in a duct ligated at its junction with the distal, acinar-containing regions of the gland (open squares), particle speed was one-tenth as fast and was not responsive to stimulation by either forskolin or carbachol.

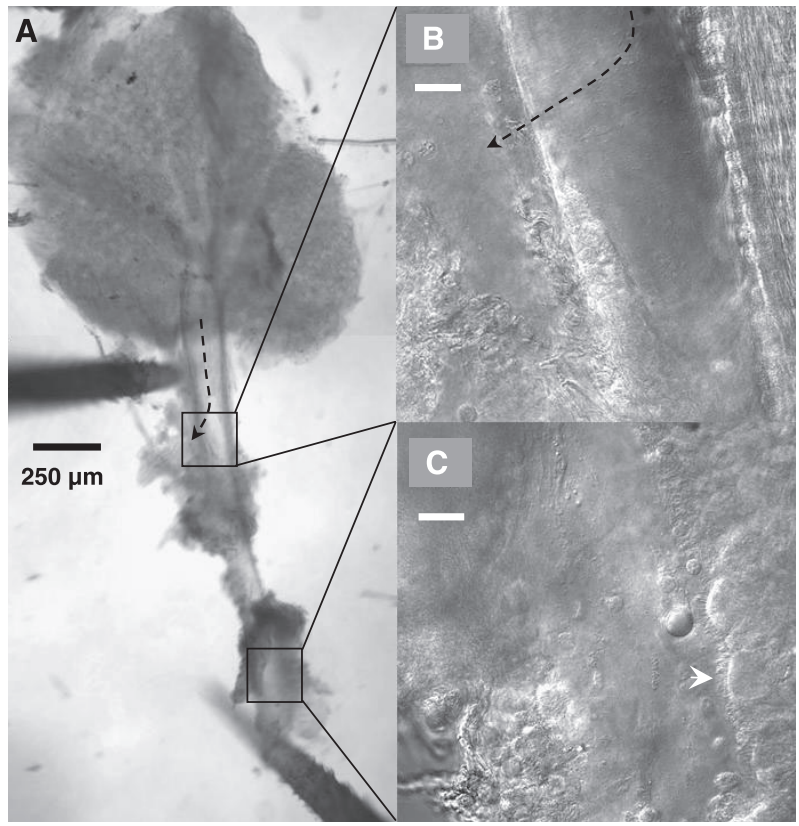

Fig. 8. Mucus movement in a nicked duct. *A*: view of isolated pig gland at low power. At the region of the upper box, a hole was made in the duct. The lower boxed region shows the duct orifice and some surrounding surface epithelium that was dissected free. *B* and *C*: the boxed regions are shown at higher magnification using DIC optics. In the supplementary, time-lapse DIC movies, mucus can be seen flowing from the distal regions of the gland along the duct to the hole and then exiting (dashed arrow). Mucus in the duct between the hole and the natural duct orifice at the surface was static, even though ductal cilia (arrowhead) were beating.

thixotropic gel (i.e., it develops gel strength when not sheared and liquefies when sheared). Thus, when static, such as at the end of a ligated duct, mucus gel strength should increase and resist the addition of further mucus. A similar explanation might have influenced the results of the nicked duct experiment (Fig. 8), where static mucus near the natural duct orifice was not moving even though the cilia were beating. However, even if filled with gelled mucus, the duct in this region should have expanded if fluid were being secreted by the ciliated cells, but it did not.

The ciliated portion of gland ducts contains ENaC subunits, raising the possibility that the duct may be absorptive and that increased absorption in CF ducts might contribute to CF gland dysfunction. In the present studies, we saw no evidence for volume absorption by the ducts, but we did not explicitly set out to test that hypothesis. Because of variability in particle speeds within the mucus (see below), we cannot completely eliminate the possibility of some volume absorption by the duct. However, that possibility has been addressed specifically by Joo et al. (10), who used ENaC blockers in an attempt to alter secretion rates from CF and normal glands. They were unable to rescue lost secretion from CF glands or to increase secretion from normal glands, and concluded that ENaC-mediated absorption does not occur in the ducts, possibly because ENaC is kept inactive by abundant Kunitz-type inhibitors present in gland secretions (10).

Several details from the particle-tracking observations raise points worth pursuing. Whereas tracking was adequate for the task of establishing an acinar origin for VIP/forskolin-mediated fluid secretion, we observed large variations in the size of particles and also noticed considerable heterogeneity in flow rates within the mucus: we observed "channels" of higher and lower flow rates that point to a considerable degree of structural complexity in the mucus as it is formed within the glands. It will be necessary to understand the origin and composition of the mucus particles and the different flow rates with the mucus stream if we are to fully appreciate the properties of normal and CF mucus.

The different patterns of CFTR expression revealed by different antibodies (5, 17) remain unresolved. The two sets of antibodies are directed at epitopes in different parts of CFTR that might be differentially exposed in different regions of the glands (W. Skatch, personal communication). It is possible that CFTR expression is indeed higher in ducts than in acini and that the monoclonal antibodies, which detected acinar CFTR in only about one-third of the samples (17), are simply inadequate to detect lower levels of acinar CFTR, which are nevertheless sufficient to mediate fluid secretion. A similar situation occurs in sweat glands, where very high levels of CFTR are present in the sweat duct alongside ENaC (3), where they jointly mediate salt absorption (20, 21). A lower level of CFTR is present in the sweat gland acini, where it mediates cAMP-stimulated fluid secretion that is lost is CF (2, 24). Indeed, we hypothesize that high densities of Na^+ and Cl^- channels are required to allow epithelia to absorb salt in excess of water, since the water permeability of membranes is relatively high even in the absence of aquaporins. The salt content of airway secretions has been a contentious issue (31), but in the only direct measures of ions in uncontaminated gland secretions, levels of Na^+ and Cl^- were significantly lower than bath values (9). Reduced levels of Na^+ and Cl^- were also found with bulk collections of nasal or tracheobronchial mucus after maneuvers that stimulated gland secretion (16).

We focused on just two immunolocalization studies with divergent results, but at least two other immunolocalization studies of CFTR in human airways have been carried out, and

these also support an acinar location for CFTR. In a study of normal human nasal turbinates using MAb 24–1, a mouse IgG_{2a} antibody directed against the four C-terminal amino acids of CFTR, Wioland et al. (33) observed intense staining of the apical membranes of serous cells, no staining of gland mucous cells, and weaker staining of surface epithelium. Jacquot et al. (8) applied anti-CFTR antibodies to human tracheal glands and saw immunolabeling along both the apical and basolateral plasma membranes of mucous cells and the secretory granules of serous cells. Using immunogold electron microscopy, the immunolabeling was more specifically associated with the membranes of serous cell secretory granules. A consistent result across these two studies is that staining with anti-CFTR antibodies was observed in gland serous cells.

In conclusion, forskolin stimulated acinar but not ductal fluid secretion. Because CF glands do not secrete in response to VIP/forskolin (11), we conclude that significant acinar fluid secretion is missing in such glands and is therefore by definition CFTR dependent. We also clearly observed acinar fluid secretion in response to carbachol and failed to observe ductal fluid secretion to carbachol. However, because carbachol induces significant fluid secretion in CF glands (9–12, 23, 26, 29, 32), the present studies do not allow us to make any statements about a possible role for CFTR in such secretion beyond showing that carbachol-stimulated fluid secretion does not have a ductal origin. Our results cannot eliminate a very minor component of ductal modification of fluid volume and do not address the possibility of ductal modification of fluid composition.

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