Mucus Secretion from Single Submucosal Glands of Pig: Stimulation by Carbachol and Vasoactive Intestinal Peptide

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SUMMARY

Secretion rates of > 700 individual glands in isolated tracheal mucosa from 56 adult pigs were monitored optically. ‘Basal’ secretion of \(0.7 \pm 0.1 \text{nml-min}^{-1}\text{gland}^{-1}\) was observed 1-9 hr post-harvest, but was near zero on day 2. Secretion to carbachol (10 \(\mu\text{M}\)) peaked at 2-3 min and then declined to a sustained phase. Peak secretion was \(12.4 \pm 1.1 \text{nml-min}^{-1}\text{gland}^{-1}\); sustained secretion was \(~1/3\) of peak secretion. Thapsigargin (1 \(\mu\text{M}\)) increased secretion from \(0.1 \pm 0.05\) to \(0.7 \pm 0.2 \text{nml-min}^{-1}\text{gland}^{-1}\); thapsigargin did not cause contraction of the \textit{trachealis} muscles. Isoproterenol and phenylephrine (10 \(\mu\text{M}\) each) were ineffective, but vasoactive intestinal peptide (1 \(\mu\text{M}\)) and forskolin (10 \(\mu\text{M}\)) each produced sustained secretion of \(1.0 \pm 0.5\) and \(1.7 \pm 0.2 \text{nml-min}^{-1}\text{gland}^{-1}\) respectively. The density of actively secreting glands was \(1.3/\text{mm}^{2}\). Secretion to either carbachol or forskolin was inhibited (~50%) by either bumetanide or \(\text{HCO}_3^{-}\), removal, and inhibited ~90% by the combined treatments. Mucus secreted in response to carbachol or forskolin was acidic by ~0.2 pH unit relative to the bath and remained acidic by ~0.1 pH unit after bumetanide. The strong secretory response to VIP, the acidity of \([\text{cAMP}]_i\)-stimulated mucus and its inhibition by bumetanide were unexpected.
INTRODUCTION

Submucosal glands play an important role in defending the upper airways from inhaled pathogens and irritants. The glands are under autonomic control and can be induced to secrete by stimulating various reflex pathways or by local mediators (1-13) for review see ref (14). The resulting mucus secretion can be copious, transiently increasing the depth of mucus on the airway surface to almost 80 µm (15). Mucus binds pathogens, inhibits their growth, and if all goes well, mucociliary transport carries the mucus and the trapped pathogens out of the airways.

All does not go well in diseases such as asthma, chronic bronchitis, the ciliary dyskinesias and cystic fibrosis (CF). For different reasons in each disease, mucociliary clearance is inefficient or lost, and the airways become susceptible to mucus plugging and infection (16). In the ciliary dyskinesias the mucus is normal but ciliary beating is ineffective. Individuals so affected have a chronic, productive cough that helps clear secretions, but are still susceptible to chronic bacterial infections that can in some cases destroy the lung (17). Thus, mucus stasis alone can permit chronic infection of the airways.

In cystic fibrosis, the cilia are normal but mucus clearance is abnormal (18). Mucus plugging is characteristic of CF organs as diverse as sinuses, intestines and gallbladder. In CF airways, normally benign bacteria and molds can be found in high densities within static mucous plugs, but not attached to the surface of the conducting airway epithelia (19), nor usually in the alveoli, nor systemically. These features indicate a defect in primary mucosal defenses of the conducting airways. Although long controversial, abnormally viscous primary mucus has now been shown to emanate from single submucosal glands in the bronchi of CF subjects, but not disease controls (20). Although it is certain that CF disease is caused by the loss of functional CFTR, an ion channel that conducts Cl⁻ and HCO₃⁻, the mechanism by which lost CFTR function increases mucus viscosity and the relation of increased mucus viscosity to CF lung disease has not been established. The uncertainties arise, in part, from our poor understanding of how submucosal glands work.

To help dissect the physiology of gland function, we have developed methods for assessing secretion rates and composition of single submucosal glands in sheep (21) and humans (20).
the present studies, we extend these methods to pigs, which have been shown by Ballard and his colleagues to be excellent models for studying human conducting airways (22-30). Our results complement their work with whole airways, and show that single glands respond to both ACh and VIP or forskolin, but with markedly different response profiles. Unexpectedly, the pH of mucus produced by these different agonists is equivalent, and they respond equally to inhibitors of Cl⁻ and HCO₃⁻ transport.
MATERIALS AND METHODS

Animal tracheas were harvested less than one hour post-mortem from adult Yorkshire female pigs that had been killed with pentobarbital injection after acute experiments unrelated to the present studies. Tracheas were maintained until use in ice cold Krebs-Ringer bicarbonate buffer (hereafter 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 adjusted to ~290 mOsm. 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 about 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 for optical monitoring of gland secretion. The mucosa with underlying glands was dissected free and mounted mucosa side up at the gas/liquid interface of a 35 mm, Sylgard-lined plastic Petri dish containing 2 ml KRB. The tissue surface was blotted, dried with a gentle stream of 95% O₂-5% CO₂ gas, and then partly covered with water-saturated mineral oil. The tissue was warmed to 37°C at a rate of ~1.5°C min⁻¹ (TC-102, Medical Systems Corp., Greenvale, NY) and superfused with warmed, humidified 95% O₂-5% CO₂. For bicarbonate-free experiments, all 25 mM of the HCO₃⁻ in the Krebs buffer was replaced with 1 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) + 24 mM NaCl that had been pre-gassed with humidified 100% O₂. The HEPES maintained the bath pH at 7.4 after gassing with O₂.

All pharmacological agents were diluted to final concentration with pre-warmed, appropriately gassed bath solution immediately before adding to the serosal bath via complete bath replacement. For washout the bath was replaced completely at least 5 times. During periods without stimulation the bath was replaced at 10-15 min intervals with fresh, 37°C KRB.
solution that had been constantly gassed with 95% O₂-5% CO₂. No changes in secretion rate were associated with bath changes.

**Optical measures**—The experimental setup was described previously (21). In brief, tissues covered with water-saturated oil were obliquely illuminated to visualize the spherical droplets of secreted mucus within the oil. Most digital images were captured at 1-5 min intervals with a CCD sensor mounted on a microscope to give a resolution of ~49,000 pixels per mm², for an area of ~6 mm². In a smaller number of experiments images of an area ~1.5-2 cm² were obtained directly with the macro lens of a Nikon 3 megapixel digital camera. Such experiments gave information on an area ~ 25-fold larger, but with resolution reduced to ~15,000 pixels per mm². Images were stored on disk for subsequent analysis, with volume calculated according to the formula for a sphere. Non-spherical droplets and merged droplets were excluded from analysis. In this method apparently spherical droplet volumes may be over-estimated to the extent that counted droplets wet the surface, but this effect is minor (21).

**Potential difference and pH measurements**—Microelectrodes with resistances of ~ 10 MΩ when filled with pH solution (see below) were placed within the secreted mucus droplets to measure the electrical potential difference (PD) of the proximal gland lumen relative to a Ag/AgCl electrode in the bath. The electrode was connected to a microelectrode amplifier (Getting Instruments) having an input impedance >10¹² Ω. In this circumstance the PD should result primarily from the epithelium of the ciliated and collecting ducts, because the contact between the surface epithelium and the mucus is minimized by the oil layer.

To measure pH, appropriate ion-selective electrodes were constructed (31) with slight modifications. LA16 glass capillaries (Dagan) were used for pH electrodes. After pulling, the tips of the ion sensing electrodes were broken back to an opening of 10-15 μm and the interiors silanized with in N, N-dimethyltrimethylsilylamine (Fluka). Each electrode tip was heated to >150°C for at least 5 minutes to cure the silanizing agent. The silanized electrodes were filled with electrolyte solution (31) and then the pH resin was drawn into the pipette under suction to a depth of ~ 1 mm. The resulting electrodes have a resistances of ~400 MΩ and can be used with any conventional microelectrode amplifier. When the ion-selective resin in the pH electrodes contacted the oil layer it lost its ion selectivity. To circumvent this problem, we fabricated electrodes in which the pH sensitive resin was recessed slightly in the tip, which was then filled
with a tiny amount of electrolyte solution. These worked, but were difficult to fabricate and were often unstable. Therefore, most pH measurements were made without oil in chambers sealed except for a slight opening for the electrode. The chambers were superfused with water saturated gas consisting of 95% O₂-5% CO₂. Equivalent results were obtained with the two methods

**Reagents**—All compounds were obtained from Sigma unless otherwise indicated, and were maintained as stock concentrations. Stock solutions in deionized water were made for, carbachol, isoproterenol, phentolamine, phenylephrine and propranolol (all at 10 mM) and vasoactive intestinal peptide (VIP, 0.1 mM). Bumetanide (0.1 M) was dissolved in an alkaline solution. Other stock solutions were indomethacin (10 mM) in ethanol and forskolin, atropine (10 mM), and thapsigargin (1 mM) in dimethylsulfoxide (DMSO). To fabricate pH sensitive electrodes we used Hydrogen Ionophore II Cocktail II (Fluka).

**Statistics**—Data are means ± SEM unless otherwise indicated. Student’s \( t \)-test for paired or unpaired data was used to compare the means of different treatment groups. The difference between the two means was considered to be significant when \( P < 0.05 \).

**RESULTS**

Our results are based on sampling >700 single glands from 54 pigs. We monitored on average 5 glands (range 1-11) per small format experiment (~300 glands), and ~100 glands per large format experiment (~400 glands).

**Pig glands secrete basally**—Basal secretion was measured 1-9 hr post-harvest for 182 glands from 18 pigs (Fig. 1). The mean basal secretion rate averaged over a 20 min period for each gland was 0.7 ± 0.1 nanoliter·min⁻¹·gland⁻¹ (nl·min⁻¹·gl⁻¹) and this rate was consistent over the 9 hr time period. Secretion rates varied considerably among individual glands. Within a contiguous 6.25 mm² area of tracheal tissue containing 9 basally secreting glands, the fastest gland secretion rate was 38 times the slowest rate, \( i.e. \ 2.3 \) and 0.06 nl·min⁻¹·gl⁻¹ (Fig. 1a). Inter-gland differences greatly exceeded differences in average basal secretion rates among pigs, which varied only about 6-fold, from 0.25 ± 0.06 (10 glands) to 1.55 ± 0.28 (7 glands) nl·min⁻¹·gl⁻¹.
Basal secretion was not inhibited by 1 µM indomethacin, 0.1 µM tetrodotoxin (TTX), 10 µM atropine, or 10 µM propranolol.

The mean basal secretion rate in each preparation declined over time post-harvest (Fig. 1b). Compared with tissues tested from 1-9 hr post-harvest, basal secretion rates measured 18-31 hr post-harvest had declined to ~ 38 % of the initial values (0.27 ± 0.05. nl·min⁻¹·g⁻¹, 129 glands in 16 pigs, P < 0.001). The number of basally secreting glands declined slightly over the same time period, from 4.7 to 3.9 glands per experiment (n.s.). The cause of basal secretion might be a response to a combination of trauma from the dissection, mechanical stimulation from the tissue preparation, or temperature fluctuations as reported previously (26).

Gland secretion was easily distinguished from surface cell secretion (Fig. 1c,d). When compared with glands, surface cells produced fluid droplets that were smaller and more closely spaced. Surface cell secretion was observed variably, both among pigs and in different regions of the same trachea. It did not appear to respond to carbachol or forskolin (Fig. 1d) and did not decline in magnitude with time after harvest, as did spontaneous gland secretion. Surface cell secretion is an active process and is not leakage through a damaged epithelium, because there is no hydrostatic pressure to move fluid into the oil layer, (the fluid layer is ~ 1 mm below the surface layer of the epithelium). Consistent with that, blue dextran placed in the bath did not appear in these droplets, nor did fluid accumulate in the oil when small holes were deliberately made in the epithelium. Surface secretion were not observed when the preparation was kept cold. We hypothesize that surface secretion originates from goblet cells, but have not yet investigated this type of secretion further.

Carbachol stimulated gland secretion—Glands secreted copiously after bath application of the cholinergic agonist carbachol (10 µM). A dose-response relation was not determined, but in a small number of experiments we determined that 1 µM was clearly sub-maximal while increasing the dose from 100 µM did not further increase the response. Single gland secretion to carbachol included a short-latency, transient peak followed by sustained secretion that was ~1/3 of the peak response (Fig. 2a, b). As shown, differences were observed in the temporal patterns of secretion among glands.
The mean peak secretion rate to carbachol was 12.4 ± 1.1 nl·min⁻¹·gl⁻¹ (194 glands, 29 pigs) when measured at 1 min intervals. Rates of carbachol-stimulated peak secretion varied ~10 fold among adjacent glands in the same preparation i.e., from 55.8 to 5.2 nl·min⁻¹·gl⁻¹ in one tissue preparation containing 5 glands in an area of 6.25 mm², but the mean peak secretory response to carbachol across pigs varied only 3.7 fold. The mean latency to peak was 2.8 ± 0.1 min when measured at 1 min intervals (194 glands, 29 pigs Fig. 2c).

Following the transient peak response, secretion declined to a smaller value that was sustained for as long as we were able to follow it. For 54 glands in 12 pigs, sustained secretion to carbachol, defined as secretion for at least a 5 min period occurring 15-60 min post stimulation, was 2.4 ± 0.2 nl·min⁻¹·gl⁻¹, vs. a mean peak value of 7.8 ± 0.7 nl·min⁻¹·gl⁻¹ in those same glands. Sustained secretion was measured for fewer glands because mucous bubbles from adjacent glands often merged within minutes after stimulation with carbachol, and thus could not be followed further.

Responses to carbachol were observed for 100% of basally secreting glands. In addition, carbachol stimulated secretion from glands for which no basal secretion had been observed in at least a 20 min observation period prior to stimulation, suggesting that most glands probably respond to carbachol.

In contrast with the gradual decline in basal secretion on the second day after harvest, the responses stimulated by carbachol, both peak and sustained, were unchanged during a period of at least 47 hr post harvest (Fig. 2d, e).

Thapsigargin stimulated small but sustained secretion—Thapsigargin inhibits Ca²⁺ uptake by the endoplasmic reticulum and elevates cytosolic Ca²⁺ unaccompanied by rises in other intracellular signals typically involved in Ca²⁺-mediated responses (32). When applied to the bath at high concentrations (1 µM), thapsigargin produced modest but sustained secretion in ~50% of glands after a variable latency of 10-50 min (Fig. 3). In 25 responding glands in 4 pigs, thapsigargin increased the mean sustained secretion rate 7-fold, from 0.1 ± 0.05 to 0.7 ± 0.2 nl·min⁻¹·gl⁻¹. Subsequent addition of forskolin caused an increased rate of secretion similar to that seen with forskolin alone, (1.9 ± 0.5, nl·min⁻¹·gl⁻¹, 17 glands, 2 pigs). Subsequent addition of
carbachol caused typical large and fast secretory responses. Exposure to 1 μM thapsigargin neither stimulated airway smooth muscle nor prevented the contraction induced by carbachol (Fig. 4). The small and variable responses to thapsigargin suggest that this agent will be of limited use in the analysis of gland function and the comparison of glands in control and disease states. Although thapsigargin has the highly specific molecular effect of inhibiting SERCA, the consequences of such inhibition are complex within even single cells, and even more so within a complex system such as the one we are studying [for example, see ref (33)].

Adrenergic agonists were ineffective—The α- and β-adrenergic agonists phenylephrine and isoproterenol (10 μM) had, at best, weak, inconsistent and transient effects on secretion. For 30 glands in 4 pigs, only 9 (30%) showed increased responses after isoproterenol, with peak gland secretion of 1.0 ± 0.4 nl·min⁻¹·gland⁻¹, and this response was transient, returning to baseline within 10 ~ 20 min after the treatment. As we previously reported (21), pigs and sheep, in contrast with cats, have only small responses to phenylephrine: for 33 glands in 6 pigs, the mean peak response for 13 (39%) responding glands was 1.3 ± 0.3 nl·min⁻¹·gland⁻¹, and this response was also transient.

VIP and Forskolin stimulated sustained secretion—Submucosal glands are innervated by peptidergic nerves, and Calu-3 cells, a serous cell model, contain functional VIP receptors (34). In addition, prior work showed good secretory response to forskolin from bronchial segments of pigs (30). Therefore we looked for response to both these agents, which elevate [cAMP]. In contrast with the transient responses to isoproterenol, pig glands showed sustained secretion to VIP and forskolin. The response profiles to these agents, which elevate [cAMP], differed distinctly from the responses to carbachol. They lacked a sharp, early peak, requiring 10-15 min to reach a maximum rate that was then sustained for at least 1 hr.

Secretion rates for 8 glands in response to VIP are plotted in Fig. 5. At 20 hr post-harvest, VIP (1 μM) increased the secretion rate in 3 basally secreting glands and initiated secretion in 5 other glands. Secretion peaked for all glands at the 10 min time point and remained elevated for at least 45 min in all but one gland. The mean peak and sustained secretion rates to VIP were 1.15 ± 0.17 and 1.04 ± 0.51 nl·min⁻¹·gland⁻¹, respectively, and the mean latency to peak was 12 ± 4.5 (SD) min (35 glands in 5 pigs). These figures exclude 1 trachea with a “wet” appearance in
which responses to VIP and carbachol were both unusually small. Response rates and profiles to forskolin (10 µM, Fig. 6) were similar but somewhat larger than were those to VIP. The mean peak and sustained responses for 231 glands measured in 26 pigs was 2.29 ± 0.37 and 1.69 ± 0.22 nl·min⁻¹·g⁻¹, respectively, but the distribution showed marked kurtosis and was positively skewed. The mean latency to peak was 17.8 ± 9.6 (SD) min for 43 glands from 6 pigs, but this figure is skewed by occasional late peaks that were only slightly higher than the rates reached at 10-15 min.

All basally secreting glands increased their rate of secretion in responses to VIP or forskolin, and some inactive glands were recruited. Responses to forskolin (Fig. 6d) and VIP (data not shown) did not decline for at least 24 hr post harvest. When VIP was added after forskolin, it did not cause additional secretion. When forskolin was added after VIP, it increased secretion in 4 of 11 glands in which it was possible to follow the response. The larger response to forskolin may simply mean that 1 µM VIP was not maximal; we did not try larger amounts because of cost. The magnitude of responses in glands that responded to both carbachol and forskolin were positively correlated (Fig. 6e), with the transient peak responses to carbachol being ~6-fold greater than the maximal response (which was ≈ sustained response) to forskolin. As expected from the above relationship, and the much faster latency of the response to carbachol, we observed a small proportion of glands that responded to carbachol but not to forskolin.

Secretion was inhibited by bumetanide and HCO₃⁻ replacement—We used bumetanide to block the Na⁺–K⁺–2Cl⁻ cotransporter NKCC1 in an attempt to reduce or eliminate Cl⁻-mediated fluid transport. We replaced HCO₃⁻ with HEPES and gassed with air to eliminate HCO₃⁻-mediated fluid transport. Carbachol-stimulated secretion was inhibited by ~50% by each inhibitor, and reduced by 90% when they were used in combination (Fig. 7a). Forskolin-stimulated secretion showed a similar pattern (Fig. 7b).

Density of active glands—The numbers of secreting glands were counted in 6 large format experiments from 4 pigs (e.g. Fig. 8), giving the values shown in Table 1. The overall average was 1.3 secreting glands/mm². This figure is similar to figures based on anatomical methods.
The method will always underestimate the number of glands because it misses refractory glands, but it can overestimate density because the epithelium retracts.

**Potential difference (PD) and pH measurements**—As described in Methods, the PD across the epithelium of the gland can be measured by placing an electrode in the mucus bubble under oil and a reference electrode in the bath. With repeated measures of this kind, for secretions stimulated by forskolin or carbachol, the PD did not differ significantly from zero. We also failed to measure a PD when recording without oil. The lack of a PD is consistent with an earlier report that gland secretion might be electrically silent (37), but is inconsistent with a report that ENaC subunits are expressed in the ducts of submucosal glands (38,39), where they would be expected to participate in electrogenic absorption of Na⁺. An insignificant PD could also mean that the duct is normally electrically leaky or was shunted by epithelial damage.

Measurement of pH with ion-sensitive microelectrodes revealed that mucus pH was acidic relative to the bath under all conditions tested (Fig. 9). For basal secretion, the mean pH was more acidic by $0.15 \pm 0.07$ pH units (27 glands, 5 pigs), for carbachol-stimulated secretion, the mean pH was more acidic than the bath by $0.20 \pm 0.03$ pH unit (22 glands, 5 pigs), and for forskolin stimulated secretion, the mean pH was more acidic than the bath by $0.17 \pm 0.03$ pH unit, 40 glands, 7 pigs. Mucus secretion stimulated by forskolin from glands pre-treated with bumetanide was also more acidic than the bath by $0.08 \pm 0.02$ pH units, but this value was significantly more alkaline than forskolin alone. These figures compare with a relative acidity of 0.4 pH units measured with the pH indicator BCECF in normal and CF human mucus stimulated with carbachol (20). This could represent a species difference or could arise from the different methods used. Regardless, the significant finding was the near-identical pH of mucus produced by the Ca²⁺-elevating agent carbachol and the [cAMP]ₐ-elevating forskolin. The identical pH produced by these different agonists is as unexpected as the identical pH values found for mucus from control and cystic fibrosis, and leads us to speculate that the final pH of the mucus is being homeostatically controlled by post-secretion processes (see discussion).
DISCUSSION

The present studies apply improved optical methods for studying the dynamics of mucus secretion from single submucosal glands of pigs. The main advance of this work over our prior studies of sheep (21) and humans (20) is a description of the properties of VIP and forskolin-mediated secretion. Rates of sustained secretion to these [cAMP]_i elevating agents were 50-80% of the sustained response to carbachol; figures in good agreement with a prior study of forskolin-stimulated mucus secretion from pig airway segments (30). VIP and forskolin did not cause muscle contractions (40), and lacked the short latency peak response seen with carbachol.

Prior studies of VIP effects on gland secretion—Vigorous, sustained secretion to VIP was not predictable from prior studies of the effects of VIP on submucosal gland secretion, which provide differing views of effects. In humans, in vitro release of mucus and lysozyme by explants of human bronchial mucosa and mucous and serous cells of the submucosal glands was slightly inhibited by VIP (41) and it was concluded that VIP has little effect on gland secretion (42). In ferrets, release of 35S-labeled macromolecules from tracheal explants was increased by VIP with a k_{1/2} of ~10 nM and a maximal response at ~ 1 µM. Because no changes were observed in electrical properties of tissues in Ussing chambers, it was concluded that VIP stimulates release of sulfated macromolecules from the submucosal glands without stimulating ion transport (43). Subsequent studies in ferrets confirmed a significant (180% increase) in 35S-labeled macromolecules by VIP, and showed that VIP led to degranulation of serous cells (44). In isolated submucosal glands from cats, VIP produced a dose-dependent increase in [3H]glycoconjugate release of up to 300% of controls (maximal at 1 µM), without increasing tension in the gland myoepithelial cells (40). Our findings contradict some of the conclusions of these prior studies, but not necessarily the results. Instead, we conclude that measurements of macromolecular secretion and I_{sc} measurements are poor predictors of bulk fluid flow from glands.
Comparison with results from studies of Calu-3 cells—The Calu-3 cell line shares a large set of biochemical features with gland serous cells (45) and is presently the best available model for them (46-48). In I_sc experiments, Calu-3 cells secrete both Cl⁻ and HCO₃⁻. Secretion produced by elevated [cAMP]ᵢ is HCO₃⁻-rich, as indicated by its resistance to bumetanide and the lack of a net flux of Cl⁻. Of more relevance, when stimulated with forskolin in open circuit, Calu-3 cells generate an apical pH of ~7.8 (49). Stimulation with agents that elevate Ca²⁺ activates basolateral K⁺ channels, hyperpolarize the cell and increase the driving force for Cl⁻ secretion (49). Thus, Ca²⁺-dependent secretion is strongly inhibited (~80%) by bumetanide (48,50), and Ca²⁺ stimulation secretions will have a reduced HCO₃⁻:Cl⁻ ratio. They may also contain an absolutely smaller amount of HCO₃⁻ because hyperpolarization creates a less favorable gradient for the transport of HCO₃⁻ through the electrogenic, basolateral Na⁺-HCO₃⁻ cotransporter, with the magnitude of this effect depending upon the stoichiometry of the transporter (49). In summary, in Calu-3 cells secretion stimulated by increased [cAMP]ᵢ has a basic pH, is resistant to bumetanide and strongly inhibited by HCO₃⁻ replacement, while secretion stimulated by increased [Ca²⁺]ᵢ is strongly inhibited by bumetanide and is predicted to have a less basic pH and to be inhibited less by HCO₃⁻ replacement.

Our findings with intact glands differ in all these respects from expectations based on Calu-3 cells. In glands, secretion stimulated by increased [cAMP]ᵢ has an acidic pH and is inhibited equally by bumetanide and HCO₃⁻ replacement, while secretion stimulated by increased [Ca²⁺]ᵢ has the same acidic pH and responds equivalently to inhibitors! Even in the presence of bumetanide, forskolin-stimulated gland secretion is acidic, although less so than without bumetanide.

The most likely explanation for the differences between Calu-3 and gland secretions is that gland serous cell secretions are only one component of gland secretions and are modified before we collect them. Glands are complex organs, and most glands operate by a two-stage mechanism in which the primary secretion is subsequently modified. The finding that the final pH values are identical for the two kinds of stimulus, as well as for normal and cystic fibrosis glands, leads us to speculate that ductal modification is homeostatic and reaches a final common pH regardless of the composition of the primary secretion.
A schematic of submucosal gland function (Fig. 10) can help frame hypotheses about gland function. The schematic, based on anatomical studies, divides submucosal glands into 4 compartments based on gross structure and cell types (51,52); the location of CFTR in serous cells is based on immunohistochemistry (53) and physiological studies of primary serous and mucous cells (54-57), and location of VIP receptors on serous cells is based on degranulation studies (44). In this model, gland mucous secretion is the joint product of serous and mucous cells. Serous cells secrete a Cl⁻/HCO₃⁻ rich fluid by CFTR-dependent mechanism in response to both cholinergic and VIP pathways. They also secrete a host of proteins and peptides including MUC7 (58) lysozyme, secretory IgA, SLPI, SP-A, and lactoferrin.

Little is known about transport mechanisms of mucous cells. We propose that mucous cells secrete the bulk of the mucin (MUC5B) molecules (58). We hypothesize that the accompanying water and ion secretions from these tubules occur via non-CFTR-dependent mechanisms, because mucus secretion is partially preserved in CF glands (20). The secretions from both types of cell are mixed and conditioned in the collecting duct and ciliated ducts. No direct evidence is available on the nature of the processes occurring in these regions of the gland, but the ciliated duct may be similar to surface epithelium, i.e. primarily absorptive and able to acidify secretions via H⁺, K⁺, ATPase (59). On the basis of pH and [Na⁺] measurements [ref. (20) and this paper], we infer that HCO₃⁻ is either absorbed or converted to CO₂ and water, that Na⁺ is also absorbed, and that either an osmolyte is added or the solution is able to remain hypotonic. This model can account for most existing results, and makes specific predictions about differences between normal and CF glands. We and others are presently developing the methods needed to support or eliminate these and related hypotheses of submucosal gland function.

Acknowledgments—We thank Dr. Jin V. Wu for expert assistance in the development of the optical methods used in this research, and Rahul S. Dhillon, Christina Y. Tseng and Tracy Hsu for data analysis. Toshiya Irokawa, Drew Hotson and Rabin Tirouvanziam provided helpful suggestions. Supported by DK51817, HL60288, and the Cystic Fibrosis Foundation.
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Fig. 1. Basal gland and surface cell secretion.  

a. Distribution of basal secretion rates for 154 glands from 16 pigs, measured from 1-9 hr post-harvest.  
b. Each symbol denotes the mean basal secretion rate over a 20-60 min period for 1-11 glands from separate tissue preparations from 23 pigs.  
c, d. Surface cell secretion was observed in ~ 20% of our preparations and was easily distinguished from gland cell secretion.  
c. is after 30 min forskolin stimulation and d. after 2 min carbachol stimulation.  
Arrows indicate selected surface cell secretions.

Fig. 2. Responses to carbachol.  

a. Volume accumulation for 3 glands from a representative experiment. Each point is the average ± SEM of independent measurements by 4 observers.  
b. Secretion rates plotted at 1 min intervals for individual glands (gland with intermediate rate omitted for clarity). Carbachol (10 µM) was present from the 20 min time point.  
c. Distribution of latencies to peak response to carbachol. Mean = 2.8 min, data from 194 glands in 29 pigs.  
d. Peak responses to carbachol as a function of time since harvest. Each point is the mean peak secretion rates of 2-10 glands from a single experiment in 23 pigs.  
e. Sustained responses to
carbachol as a function of time since harvest. Data are mean ± SEM from 9-33 glands in 3-9 pigs.

**Fig. 3. Gland secretion to thapsigargin.** In this preparation, 1 µM thapsigargin stimulated secretion in only 2 of 8 glands, all but one of which subsequently responded to 10 µM forskolin.

**Fig. 4. Response of airway smooth muscle to thapsigargin and carbachol.**  
*a.* Unstimulated segment of posterior (dorsal) trachea which contains the *trachealis* muscles. In these figures, the anterior-posterior axis of the tissues is oriented horizontally.  
*b.* No contraction observed following 60 min exposure to 1 µM thapsigargin.  
*c.* Contraction produced by subsequent 5 min exposure to 10 µM carbachol. Grid consists of 0.5 mm squares.

**Fig. 5. Gland secretion to VIP.**  
*a.* Cumulative volume secreted in each of 8 glands following stimulation with 1 µM VIP.  
*b.* Corresponding secretion rates for the glands shown in *a.* VIP (1 µM) was present from the 20 min time point.

**Fig. 6. Gland secretion to forskolin.**  
*a, b.* Segments of a large format view 0 and 50 min after stimulation with 10 µM Forskolin.  
*c.* Volume increases in 3 glands from a different experiment as a function of time after forskolin stimulation.  
*d.* Magnitude of the secretory response to forskolin as a function of time post-harvest. Each symbol represents the average of sustained secretion rate over a 5-105 min period from a separate tissue preparation from 231 glands from 26 pigs.  
*e.* Correlation between carbachol and forskolin secretion rates for individual glands. Each symbol plots the peak secretion rate to carbachol on the y axis and the peak secretion rate to forskolin on the x axis for an individual gland. Correlation coefficient = 0.81.

**Fig. 7. Inhibition of agonist-stimulated gland secretion by bumetanide (Bm, 0.1 mM) and HCO$_3^-$ replacement.**  
*a.* Secretion stimulated by carbachol.  
*b.* Secretion stimulated by forskolin. Each bar shows the % of residual secretion after inhibition by each agent alone and in combination. Data are means ± SE from 29-41 glands from 5-8 pigs. *, **Significantly different
from control, $P < 0.05$, 0.005 respectively. The combined treatment also differed significantly from either agent alone, but the differences between bumetanide or HCO$_3^-$ replacement were not significant.

**Fig. 8. Density and distribution of actively secreting glands.** An example of a preparation used to determine gland density. This method undercounts glands, because slowly secreting glands are missed at early intervals, and secretions merge at later intervals. However the tissue retracts when removed from the cartilage, and this causes an overestimate of gland density.

**Fig. 9. Mucous pH.** Each bar shows the mean pH for secretions produced by the indicated conditions. All were acidic relative to the bath, which was measured before and after each measure of secretion, averaged, and normalized to 7.4. The number of pigs and glands measured is shown in each bar. *Significantly different from other three measures $P < 0.05$.

**Fig. 10. Schematic of submucosal gland.** Four functional compartments are proposed based on prior anatomical data, with CFTR located primarily in serous cells (see text).
<table>
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<th>Glands (n)</th>
<th>Glands/mm$^2$</th>
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<td>1.3</td>
</tr>
</tbody>
</table>

**Table I. Density of active glands in pig trachea.**

For each of the conditions shown an area of trachea from ~14 to 79 mm$^2$ was imaged at 10 min intervals and the number of mucus bubbles emerging under the oil was counted.
Fig 1. **Basal gland and surface cell secretion.**  

*a.* Distribution of basal secretion rates for 154 glands from 16 pigs, measured from 1-9 hr post-harvest.  
*b.* Each symbol denotes the mean basal secretion rate over a 20-60 min period for 1-11 glands from separate tissue preparations from 23 pigs.  
*c, d.* Surface cell secretion was observed in ~20% of our preparations and was easily distinguished from gland cell secretion.  
*c.* is after 30 min forskolin stimulation and  
*d.* after 2 min carbachol stimulation.  
Arrows indicate selected surface cell secretions.
Fig 2. **Responses to carbachol and accuracy of measurements.**  

*a.* Volume accumulation for 3 glands from a representative experiment. Each point is the average ± SEM of independent measurements by 4 observers.  

*b.* Secretion rates plotted at 1 min intervals for individual glands (gland with intermediate rate omitted for clarity). Carbachol (10 µM) was present from the 20 min time point as arrows indicate.
Fig. 2c. **Distribution of latencies to peak response to carbachol.** Mean = 2.8 min, data from 194 glands in 29 pigs.
Fig. 2d. Peak responses to carbachol as a function of time since harvest. Each point is the mean peak secretion rates of 2-10 glands from a single experiment in 23 pigs.
Fig. 2e. **Sustained responses to carbachol as a function of time since harvest.** Data are mean ± SEM from 9-33 glands in 3-9 pigs.
Fig. 3. **Gland secretion to thapsigargin.** In this preparation, 1 μM thapsigargin (Tg) stimulated secretion in only 2 of 8 glands, all of which subsequently responded to 10 μM forskolin (Fsk).
Fig. 4. **Response of airway smooth muscle to thapsigargin and carbachol.** A. Unstimulated segment of posterior (dorsal) trachea which contains the *trachealis* muscles. In these figures, the anterior-posterior axis of the tissues is oriented horizontally. B. No contraction observed following 60 min exposure to 1 µM thapsigargin. C. Contraction produced by subsequent 5 min exposure to 10 µM carbachol. Grid consists of 0.5 mm squares.
Fig. 5. **Gland secretion to VIP.**  

a. Cumulative volume secreted in each of 8 glands following stimulation with 1 µM VIP.  
b. Corresponding secretion rates for the glands shown in a. VIP (1 µM) was present from the 20 min time point as shown with arrows.
Fig. 6. **Gland secretion to forskolin.** *a, b.* Segments of a large format view 0 and 50 min after stimulation with 10 µM forskolin. *c.* Volume increases in 3 glands from a different experiment as a function of time after forskolin stimulation.
Fig 6d. Magnitude of the secretory response to forskolin as a function of time post-harvest. Each symbol represents the average of sustained secretion rate over a 5-105 min period from a separate tissue preparation from 231 glands from 26 pigs.
Fig. 6e. **Correlation between carbachol and forskolin secretion rates for individual glands.** Each symbol plots the peak secretion rate to 10 µM carbachol (Carb) on the y axis and the peak secretion rate to 10 µM forskolin (Fsk) on the x axis for an individual gland. Data are 42 glands from 9 pigs. Correlation coefficient = 0.81.
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- **a.** Secretion stimulated by carbachol.  
- **b.** Secretion stimulated by forskolin. Each bar shows the % of residual secretion after inhibition by each agent alone and in combination. Data are means ± SEM from 29-41 glands from 5-8 pigs. *, **Significantly different from control, *P* < 0.05, 0.005 respectively. The combined treatment also differed significantly from either agent alone, but the differences between bumetanide or HCO_3^- replacement were not significant.
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- Basal: 5/27
- Carbachol: 5/22
- Forskolin: 7/40
- Bumetanide/Forskolin: 4/41
Fig. 10. **Schematic of submucosal gland.** Four functional compartments are proposed based on prior anatomical data, with CFTR located primarily in serous cells (see text).
Mucus secretion from single submucosal glands of pig: Stimulation by carbachol and vasoactive intestinal peptide
Nam Soo Joo, Yamil Saenz, Mauri E. Krouse and Jeffrey J. Wine

J. Biol. Chem. published online May 13, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M202712200

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