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HCO_3^- Transport in Relation to Mucus Secretion from Submucosal Glands

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Summary

The role of HCO_3^- transport in relation to fluid secretion by submucosal glands is being studied in sheep, pigs, cats and humans. Optical methods have been developed to measure secretion rates of mucus volume from single glands with sufficient temporal resolution to detect differences in minute-by-minute secretion rates among glands. The ionic composition and viscoelastic properties of the uncontaminated gland mucus are measured with a combination of ratiometric fluorescent indicators, ion-selective microelectrodes, FRAP, and a miniaturized, magnetic force viscometer. Sheep glands secreted basally at low rates, showed small, transient responses to alpha- and beta-adrenergic agonists, and large responses to a cholinergic agonist, carbachol. Peak rates and temporal patterns of responses to carbachol differed markedly among glands. To assess the contribution of HCO_3^- transport to gland secretion, we either inhibited $Na^+/K^+/2Cl^-$ cotransporter (NKCC) with bumetanide or replaced HCO_3^- with HEPES and gassed with O_2. Bumetanide caused a small, non-significant inhibition of basal secretion, but removal of HCO_3^-/CO_2 significantly reduced basal secretion almost by half. Both bumetanide and removal of HCO_3^-/CO_2 reduced carbachol-stimulated secretion significantly, with HCO_3^- removal having the larger effect: a reduction to 33% of control ($P<0.01$). The remaining secretory response to carbachol was nearly eliminated by bumetanide. Sheep mucus pH measured with ion selective electrodes was about 0.4 log more acidic than the bath. In humans, we observed the same pattern of responses to agonists and antagonists as in sheep, and observed a mucus pH of 7.0 using 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF). We hypothesize that HCO_3^- transport is important in the formation of mucus secretion, but that most HCO_3^- is scavenged before the final mucus appears at the duct opening.

Cystic fibrosis transmembrane conductance regulator's (CFTR) best understood function is as an anion channel, but increasing attention has been given to its role in HCO_3^- transport [1, 2, 3, 4, 5, 6, 7, 8, 9, 10]. By analogy with organ-specific CFTR effects on Cl^- transport [11], it seems likely that the relative importance of CFTR in HCO_3^- transport will also vary across organs. Because lung disease is by far the greatest cause of mortality among people with cystic fibrosis, it is important to determine how loss of CFTR function causes lung disease. We are testing the hypothesis that loss of CFTR alters serous cell secretion in the lungs, and the

corollary that such loss contributes to cystic fibrosis (CF) lung disease. CFTR is highly expressed in serous cells of submucosal glands [12] and the Calu-3 serous cell model secretes HCO_3^- [13, 14]. Human gland serous cells grown in culture and tested for fluid secretion under open circuit conditions showed reduced fluid secretion to all mediators [15].

However, submucosal glands are complex organs containing at least 4 distinct regions [16] and at least that many cell types, making it difficult to predict the consequences on whole-organ function from experiments with individual cell types. Therefore, we have resurrected long-neglected methods for studying whole-gland function [17], and have attempted to improve them in a variety of ways. We are refining these methods and increasing our understanding of gland function by studying tracheal glands from sheep, pigs and cats. As human tissues become available, they are studied with the best methods presently available. The key questions now being asked are: Is mucus secretion from submucosal glands altered in cystic fibrosis? If so, how is it altered and how does it contribute to CF lung disease? Answering the last question will require an understanding of how glands interact with other regions of the lung. In the context of this meeting, we present preliminary data on the role of HCO_3^- in gland mucus secretion.

Single Gland Secretion Studies: Sheep Tracheal Glands

An initial set of experiments was carried out using tracheas from Suffolk-Rambouillet sheep [18]. Isolated tracheal mucosa were mounted in a temperature controlled chamber, such that the serosa was submerged in Krebs solution while the mucosal surface was in air. The surface was cleaned, dried and coated with water-saturated mineral oil; the preparation was superfused with humidified 95% O_2 / 5% CO_2 and maintained at 37 ºC. Under these conditions gland secretions formed spontaneously into spherical bubbles that were digitally imaged at intervals allowing rates of secretion to be calculated.

Glands secreted basally at low rates (0.6 nL/min/gland) in tissues up to 9 h post-harvest and at lower rates for up to 3 days. Basal secretion was not reduced by tetrodotoxin (TTX), indomethacin, or a cocktail of blockers to adrenergic and cholinergic agonists. It was reduced only slightly and non-significantly by 100 μM bumetanide, but it was significantly reduced to about half normal rates by replacement of HCO_3^- with HEPES and simultaneous switching of gassing from 95% O_2 / 5% CO_2 to O_2.

Stimulation with mediators for alpha-adrenergic and beta-adrenergic stimulation produced only small transient responses, whereas the cholinergic agonist carbachol (10 μM) stimulated copious secretion. Because the alpha-adrenergic agonist phenylephrine had previously been found to be a potent agonist for secretion in cat airway submucosal glands [17, 19] we also assessed phenylephrine effects on gland secretions in cats, pigs and humans and found it to be effective only in cats.

In contrast, the response to carbachol was similar in all 4 species. Carbachol produced an early transient that peaked, on average, at 3 min, followed by a sustained response that continued as long as the gland was followed. Peak secretion was 16 nL/min/gland and sustained secretion was 4.5 nL/min/gland. In ventral trachea we observed an average of one secreting gland duct opening per mm^2, consistent with other estimates [20]. Therefore, the average sustained response we saw predicts a whole tissue response of about 27 $\mu L/cm^2/h$. Some glands showed oscillating secretion rates, with a period of about 0.01 Hz, similar to that previously reported for I_{SC} responses in Calu-3 cells [21, 22].

Carbachol-stimulated secretion was inhibited 56% by bumetanide, 67% by HCO_3^- replacement with HEPES, and 92% by combined treatment. These figures can't be

used to apportion secretion rates between Cl^- and HCO_3^- because inhibition of either pathway can lead to compensatory increases in the other pathway. However, they strongly suggest that HCO_3^- transport plays an important role in gland mucus secretion.

Single Gland Secretion Studies: Pig Tracheal Glands

A similar but more extensive series of studies has been carried out in pigs. Experiments are in progress and data are only partly analyzed. So far, results with pigs parallel those with sheep, with a few additional findings.

First, we have measured the potential difference in mucus bubbles relative to bath. The potential difference is usually near zero, with an occasional gland producing a tendency toward negativity of no more than -2 mV. This rules out the possibility that the gland duct epithelium is a high resistance epithelium with electrogenic reabsorption.

Second, we have measured the pH of mucus droplets secreted in response to carbachol, and find it to be about 0.3 pH units lower than the bath. Similar measures of acidic mucus pH were reported previously for the ferret tracheal secretions [23, 24].

Third, sustained mucus secretion is stimulated by forskolin (10 μM) at a rate that is similar to the sustained rate to carbachol, (1.5-3.0 nL/min/gland) but without the large transient peak seen with carbachol. Secretion stimulated by forskolin was not reduced much by replacement of HCO_3^-/CO_2 with HEPES/air, but was eliminated when bumetanide was added to the HEPES/air treatment.

Single Gland Secretion Studies: Human Tracheal-Bronchial Glands

Lung transplantation has become the intervention of last resort in several human diseases including cystic fibrosis. By arrangement with Stanford surgeons, we obtain tracheal trimmings from normal donors and excised lung tissues from CF subjects and other subjects with lung or vessel diseases. The tissues are immediately transported to the laboratory and studies of gland secretion can begin within hours. When tissues or bronchi are properly prepared, single living glands can be imaged and studied optically. More commonly, we use an approach like that developed for sheep, in which a small field of gland duct openings is studied; glands can subsequently be injected to correlate structure and function.

We observe almost the same parameters of secretion with human glands as we do with sheep and pig glands. Comparisons of secretion in CF, disease control and normal glands are just beginning. Our initial results show that secretion is qualitatively similar between CF and normal glands, both in rates of secretion from individual glands, and in the ion composition and pH of the secreted mucus. Preliminary estimates of simple viscosity using fluorescence recovery after photobleaching indicate that viscosity is elevated in CF samples [25]. Replacement of HCO_3^-/CO_2 with HEPES/air inhibited about 30-50% of carbachol-stimulated secretion in both normal and CF glands.

Our studies with humans are just beginning, and we need to do a great deal more developmental work before the method can definitively determine if a CF-normal difference in gland function plays a role in CF lung disease.

Discussion: A Role for CFTR-Mediated HCO_3^- Secretion in Submucosal Gland Secretion and CF Lung Disease?

We find that in response to a cholinergic agonist, CF glands secrete amounts of mucus that are qualitatively similar to what is observed in control subjects, and with an equivalent (acidic) pH. Preliminary measures of viscosity suggest it is increased. Both the acidic pH and the near-normal amounts of secretion are

difficult to reconcile with a simple model in which mucus is flushed from normal glands by a HCO_3^--rich fluid that is lacking in CF.

The ability of CF glands to secrete mucus means that CFTR is not an obligatory component of the secretion pathway, in spite of its rich expression in serous cells. However, it can't be concluded that secretion rates are normal (as they are, for example, for sweat secretion in people with CF), because CF glands are reported to be grossly hypertrophied, and our measures have not been corrected for gland size. In addition, our present measures have focused on rates of individual glands. If a gland fails to secrete at all, it is not included in our measures.

The apparent contradiction remains that freshly secreted, uncontaminated mucus has an acidic pH. This contradicts predictions made from Calu-3 models of serous cells. We see a large effect of HCO_3^- removal on normal gland secretion in sheep, consistent with expectations from Calu-3 cell studies, but against expectations, results from 2 subjects suggest that CF glands are similarly affected by HCO_3^- removal, and their secreted mucus has a pH indistinguishable from normal [25]. The Calu-3 model might be misleading, but another possibility is that serous cell secretions are modified as they traverse three anatomically distinct regions of the gland on their way to the airway surface. The nature of this hypothesized modification is unknown.

These initial results suggest that gland function is complex, as suggested by gland structure, and that alterations caused by lost CFTR function are either subtle, or inadequately addressed with our present methods. We think the methods we are developing will be suitable to decide between these alternatives.

Key words Bumetanide; Carbachol; Cats; Cystic Fibrosis; Human; Hydrogen-Ion Concentration; Sheep; Swine; Viscosity

Abbreviations BCECF: 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein; CFTR: cystic fibrosis transmembrane conductance regulator; NKCC: $Na^+/K^+/2Cl^-$ cotransporter

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