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Chloride secretion by cultures of pig tracheal gland cells

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Widdicombe JH, Borthwell RM, Hajighasemi-Ossareh M, Lachowicz-Scroggins ME, Finkbeiner WE, Stevens JE, Modlin S. Chloride secretion by cultures of pig tracheal gland cells. *Am J Physiol Lung Cell Mol Physiol* 302: L1098–L1106, 2012. First published February 24, 2012; doi:10.1152/ajplung.00253.2011.— Malfunction of airway submucosal glands contributes to the pathology of cystic fibrosis (CF), and cell cultures of CF human airway glands show defects in Cl^- and water transport. Recently, a transgenic pig model of CF (the CF pig) has been developed. Accordingly, we have developed cell cultures of pig airway gland epithelium for use in investigating alterations in gland function in CF. Our cultures form tight junctions (as evidenced by high transepithelial electrical resistance) and show high levels of active anion secretion (measured as amiloride-insensitive short-circuit current). In agreement with recent results on human airway glands, neurohumoral agents that elevate intracellular Ca^{2+} potently stimulated anion secretion, while elevation of cAMP was comparatively ineffective. Our cultures express lactoferrin and lysozyme (serous gland cell markers) and MUC5B (the main mucin of airway glands). They are, therefore, potentially useful in determining if CF-related alterations in anion transport result in altered secretion of serous cell antimicrobial agents or mucus.

airway mucous gland; cystic fibrosis; anion secretion

IN HUMAN TRACHEA and main stem bronchi, the volume of mucus in glands has been estimated as 50 times that in goblet cells of the surface epithelium (28). There is about one gland per square millimeter of airway surface (5, 34). With maximal secretory rates of ~20 nl·gland^{-1}·min^{-1} (6, 35), glands can therefore add liquid to the airway lumen at up to 120 μl·cm^{-2}·h^{-1}. Absorption by the surface epithelium is ~5 μl·cm^{-2}·h^{-1} (19). Acutely, therefore, glands can have much more striking effects on the volume and composition of airway surface liquid than can the surface epithelium (37).

Several lines of evidence suggest that altered gland secretion is a major contributor to the airway pathology of cystic fibrosis (CF). Airway glands are hypertrophied in CF (1, 16) and fail to secrete in response to elevation of intracellular cAMP (6, 21). cAMP-dependent secretion of Cl^- and water by primary cultures of human airway glands is also essentially abolished in CF (20, 39). It has been suggested that these changes may lead to a deficiency in gland-derived antimicrobial agents in airway surface liquid, thereby promoting the bacterial colonization characteristic of this disease (6). Furthermore, gland secretions are abnormally viscous in CF (18), a finding that could account for reduced mucociliary transport in this disease (41). Finally, mouse airways are almost completely devoid of mucous glands (5), and transgenic mouse models of CF fail to replicate the airway pathology of human disease (14).

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Cultures of human airway glands have proved useful in demonstrating secretory defects in CF (20, 26, 39). Use of such cultures has been hindered, however, by the scarcity of postmortem or postsurgical CF tracheas. Immortalized cell lines have been developed to circumvent this problem (13), but they generally fail to form tight junctions, and, therefore, their use in studies of epithelial function is limited. Recently, a transgenic pig model of CF has been developed (29). Unlike the mouse, the number of airway glands in the pig is similar to that in humans (5, 23), and the CF pig shows airway pathology closely resembling that of human CF patients (27). These similarities between the pig and human and the large size and ready availability of the pig make it ideally suited for the generation of primary cultures of airway glands for studies on their functional alterations in CF. Here, we describe a culture technique that produces a large quantity of primary cultures of pig tracheal glands that form tight junctions and show active transepithelial anion secretion. The effects of neurohumoral agents on anion secretion by these cultures agree well with results obtained from human and pig airway glands (21, 23) and primary cultures of human glands (12). Finally, the expression of lactoferrin and lysozyme by our cultures will permit us to test the hypothesis that altered anion secretion in CF leads to reduced secretion of serous cell antimicrobial agents.

MATERIALS AND METHODS

Tracheas from young adult pigs (~250 lb body wt, 4–6 mo old) of either sex were obtained from the Meat Laboratory (a federally inspected meat-processing plant at the University of California, Davis). They were stored for up to 2 days in PBS at 4°C; the duration of storage had no discernable influence on the quality of the resulting cultures. Tracheas (~20 cm long × 2 cm diameter) were split open longitudinally and pinned out in a dissecting tray. The surface epithelium was scraped off using a sterile plectrum cut from the wall of a plastic beaker, and the mucosal surface was washed with PBS to remove any residual scrapings. Forceps and a scalpel were used to dissect the epithelium-free collagenous submucosal tissues from the underlying cartilage rings and smooth muscle, and the tissues were chopped with scissors into small (~1–5 mm^3) fragments. These were put into a trypsinizing flask containing 25 ml of Hanks' balanced salt solution containing collagenase (500 U/ml), hyaluronidase (200 U/ml), deoxyribonuclease (1 U/ml), BSA (1 mg/ml), and HEPES (10 mM) and incubated on an orbital shaker at 37°C and 150 rpm for ~2.5 h. The resulting suspension of glands and collagen fibrils was decanted from the remaining large fragments of tissue into a preweighed 50-ml cell culture tube and centrifuged for 10 min at 2,000 *g*. The pellet was suspended in 50 ml of "5% FCS," a 50:50 mixture of DMEM and Ham's F-12 medium containing 5% FCS, and centrifuged again. The supernatant was aspirated, and the tube was reweighed to obtain the weight of the pellet. The average weight of the pellets was 0.46 ± 0.10 g, and the pellets were suspended at ~20 mg/ml in 5% FCS and dispensed into T75 (10 ml) or T25 (3.3 ml) flasks that had

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been precoated with human placental collagen (HPC) (7). In preliminary experiments, we coated flasks with a number of other collagens, but none gave better attachment and growth than HPC.

Cells were grown in one or other of the following media: 5% FCS, as described above; Gray's medium, a hormonally defined medium used with surface epithelial cells of human trachea, the components of which are listed elsewhere (31); EGF medium, 50:50 DMEM-F-12 medium supplemented with epidermal growth factor (EGF, 20 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (0.5 μg/ml), triidothyronine (20 ng/ml), retinoic acid (5×10^{-8} M), BSA (2 mg/ml), and 0.1% Ultroser G (USG; Pall Biosepra, Cergy, France); EGF-free medium, similar to EGF medium, but with EGF removed; and 2% USG, 50:50 DMEM-F-12 medium with 2% USG.

On the day after plating, flasks were rinsed four times with PBS to remove debris and unattached acini, and fresh medium was added. In preliminary experiments, some cells were maintained in 5% FCS, while others were incubated in Gray's medium, EGF medium, or EGF-free medium. Growth was clearly best in EGF medium, which was used in all further experiments to expand cells in flasks prior to plating on inserts. The cells were maintained in EGF medium until ~80% confluent, i.e., for 2–5 days after plating. Then, to remove fibroblasts, the flasks were treated with 2–3 ml of 0.1% trypsin in Ca^{2+}-free PBS for 1–3 min at room temperature. Released cells were discarded, the flask was rinsed with PBS, and trypsinization was resumed at 37°C. Once all cells had detached (~15 min), the reaction was terminated by addition of 5% FCS, and the cells were pelleted at 2,000 *g* for 10 min. A cell count was performed, and the cells were suspended at 2×10^6/ml, and 10^6 cells were plated onto one of four types of insert of ~1 cm^2 area: opaque Transwell (catalog no. 3401, Corning, Corning, NY), Millicell-HA or Millicell-CM (Millipore, Billerica, MA), or Cyclopore (Falcon no. 353180, BD Biosciences, San Jose, CA). Opaque Transwell inserts are made from polycarbonate, Cyclopore inserts from polyethylene terephthalate, Millicell-HA inserts from mixed cellulose esters, and Millicell-CM inserts from hydrophilic polyfluorotetraethylene. One milliliter of 5% FCS was added to the serosal side of each insert. All inserts were precoated with HPC. Approximately 24 inserts were obtained per trachea.

On the day after the cells were plated, the medium was changed. Some cells were maintained continuously in 5% FCS. Others were grown in 2% USG, EGF medium, or EGF-free medium. Cells were grown with their apical surfaces immersed in medium or with an air-liquid interface. In the former condition, when medium was changed, it was added to the inside (0.5 ml) and outside (1 ml) of the insert (apical and basolateral aspects of the cells). For air-interface feeding, medium was added to the outside of the insert only, and once the cells became confluent, no more than a thin film of liquid was present on the mucosal surface. Medium was changed every 1 or 2 days. Transepithelial voltage, an indicator of transepithelial active ion transport, and transepithelial electrical resistance (R_{te}), an index of the formation of tight junctions, were recorded every 1–2 days using a chopstick voltmeter, as described elsewhere (31). When R_{te} became >100 Ω·cm^2 (generally 2–5 days after plating), cells were studied further in conventional Ussing chambers, as described elsewhere (38). The filters were cut out from Transwell and Cyclopore inserts and mounted between Lucite half chambers, the contact faces of which had been smeared with silicone grease to minimize edge damage. Millicell-HA and Millicell-CM inserts were mounted intact in Ussing chambers of a slightly different design. The tissues were bathed in bicarbonate-buffered Krebs-Henseleit solution bubbled with 95% O_2-5% CO_2 at 37°C. The short-circuit current (I_{sc}) required to clamp transepithelial voltage to zero was recorded continuously. R_{te} was determined from the current deflections in response to brief voltage pulses across the tissue (38). In the text, the age of the cells is given as the number of days after they were plated onto inserts. When Ussing chamber experiments were completed, cell sheets were cut in half. One half was put in 0.1 N NaOH for analysis of protein and DNA content. Cell protein was determined using bicinchoninic acid (32). After neutralization with 0.1 N HCl, DNA was determined with bisbenzamide (4) or from the difference between absorbance at 260 nm and absorbance at 280 nm, determined with a standard UV spectrophotometer (Ultrospec 2100 pro, Biochrom, Cambridge, UK).

The other half of the insert was used for conventional light microscopy, as described elsewhere (38), or for fluorescence confocal microscopy. For the latter, cell sheets were fixed for ≥30 min in buffered formalin (10%) and stored in 0.1 M phosphate buffer (pH 7.4). Cell sheets were heated for 30 min in 0.1 M sodium citrate buffer to effect antigen retrieval and then treated with methanol for 10 min at 4°C for permeabilization. In our initial experiments, we stained separately for the individual proteins. After blocking with 5% normal goat serum in Tris-buffered saline for 1 h, the cell sheets were incubated overnight at 4°C with rabbit anti-human polyclonal antibody to MUC5B (1:100 dilution; H-300, Santa Cruz Biotechnology, Santa Cruz, CA), lysozyme (1:30 dilution; A48, Biomeda, Foster City, CA), lactoferrin (1:30 dilution; H887, Accurate Chemical, Westbury, NY), or mouse anti-human MUC5AC (1:100 dilution; 45M1, Santa Cruz Biotechnology). The secondary fluorescent conjugated antibody goat anti-rabbit Cy3 (1:500 dilution; Jackson ImmunoResearch, West Grove, PA) was used to detect polyclonal rabbit primary antibodies. Secondary fluorescent conjugated goat anti-mouse Alexa 647 (Invitrogen, Carlsbad, CA) was used for MUC5AC. After exposure to antibodies, cell sheets were treated with Yo-Pro iodide (1:500 dilution, Invitrogen) to stain nuclei. Matching negative controls were generated using rabbit or mouse IgG primary antibodies. Filters were mounted in antifade reagent. Cell sheets were imaged on a confocal microscope (model L510, Zeiss); *z* stacks (1 μm between scans) were taken of each field, with an *x–y* field size of 230 × 230 or 320 × 320 μm. Later we costained for lactoferrin, lysozyme, and MUC5B or for MUC5B and MUC5AC. For the triple-stain experiments, cells were treated with rabbit anti-human lactoferrin or rabbit anti-human lysozyme (1:100 dilution) and incubated with goat anti-rabbit Cy3 (1:500 dilution). The cells were reblocked in serum and exposed to the other of rabbit anti-human lactoferrin or anti-human lysozyme (1:100 dilution) and then to goat anti-rabbit Cy5. The cells were again blocked in serum and incubated with rabbit anti-human MUC5B (1:100 dilution) and then with goat anti-rabbit Alexa 488 (1:500 dilution). Similar results were obtained whether we stained first for lactoferrin or for lysozyme. Cells dual-stained for MUC5B/AC were incubated with rabbit anti-human MUC5B (1:100 dilution) and then with goat anti-rabbit Cy3 (1:500 dilution). The cells were reblocked in serum and exposed to mouse anti-human MUC5AC (1:100 dilution) and goat anti-mouse Alexa 647 (1:500 dilution). Nuclei were stained with 4,6-diaminido-2-phenylindole (1:500 dilution). Counting was done just below (2–5 μm) the apical membrane. As a control for the triple staining, cells were exposed to normal rabbit IgG control (R & D Systems, Minneapolis, MN) and counterstained with the secondary antibodies Cy3, Cy5, and Alexa 488 sequentially, as described above. Similarly, to control for the MUC5B and MUC5AC costaining studies, cells were exposed to normal rabbit IgG and normal mouse IgG control (R & D Systems) and then to the antibodies Cy3 and Alexa 647, as described above. At the baseline gain settings used, the IgG controls were uniformly negative. For quantification, cell sheets were imaged on a confocal microscope (C1si Spectral, Nikon, Tokyo, Japan); *z* stacks (1 μm between scans) of each field were obtained, and the number and average size of nuclei were counted using Fuji Image Processing and Analysis Software in Java (http://fiji.sc/wiki/index.php/Fiji), with an *x–y* field size of 230 × 230 or 320 × 320 μm. The number of positive cells for any given protein was also counted in each stack. Data for each staining combination were obtained from four randomly selected fields from each cell sheet.

Released lysozyme was measured from the increase in fluorescence upon digestion of fluorescein-labeled *Micrococcus lysodeikticus* cell walls (Enzchek kit, Molecular Probes, Eugene, OR). PBS (800 μl) was placed on the mucosal surface of cell sheets, and a 100-μl sample was removed immediately thereafter for estimation of lysozyme

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content. After 24 h, another 100-μl sample was taken, and the mucosal liquid was collected and placed in a preweighed vial, which was reweighed to give the volume remaining, because there is some loss of volume due to evaporation (even though the incubator is supposedly at 100% humidity). Of the 800 μl added, ~300 μl were lost to evaporation (i.e., ~400 μl were present at the end of the experiment). From the volume of medium present at the start and end of the experiment and the lysozyme concentrations in the respective samples, the amount of lysozyme secreted was calculated.

Cultures of surface epithelium were generated as described elsewhere (38) and subjected to the same Ussing chamber protocol used for the gland cultures. They were grown in EGF medium, 2% USG, or 5% FCS.

All media contained penicillin (10^5 U/l), streptomycin (100 mg/l), gentamicin (100 mg/l), and amphotericin B (Fungizone, 2.5 mg/l). Except where specified, all chemicals were obtained from Sigma (St. Louis, MO). Pharmacological agents were added as 1:100 or 1:1,000 dilutions of stock solutions made up in water, ethanol, or DMSO. Addition of vehicle was without effect on any of the properties measured.

Values are means ± SE. Student's *t*-test was used to test for statistical differences between means; $P < 0.05$ was considered significant.

RESULTS

Electrophysiology: standard protocol. After I_{sc} and R_{te} had stabilized, cells were treated with amiloride (to abolish active Na^+ absorption) and then sequentially with forskolin (to activate cAMP-dependent anion secretion) and methacholine and ATP (to stimulate Ca^{2+}-dependent anion secretion). We then added $CFTR_{inh}$-172, a blocker of CFTR, followed by flufenamic acid (FFA), a blocker of Ca^{2+}-activated Cl^- channels (CaCC). A typical record for cells grown with an air interface in EGF medium on a Transwell insert is shown in Fig. 1, although similar results were obtained with several other combinations of medium and insert. As shown in Fig. 1, amiloride had no effect on I_{sc} (as was usually the case), and forskolin resulted in transient and sustained increases in I_{sc} (although in the great majority of tissues, the transient response was absent). Methacholine produced a rapid transient increase in I_{sc} followed by a rapid and then a slow decline toward baseline (see below). The maximal change induced by methacholine was considerably greater than that induced by forskolin. ATP produced a transient response similar in magnitude to that produced by methacholine. FFA was a much more potent inhibitor of I_{sc} than was $CFTR_{inh}$-172. Any residual I_{sc} was abolished by ouabain.

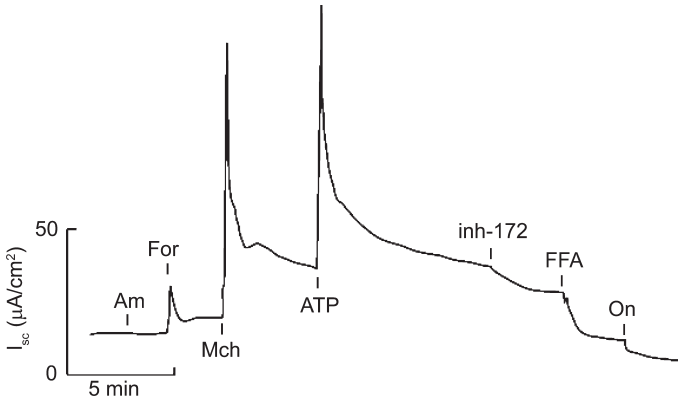

Fig. 1. Standard Ussing chamber protocol. Tissues were treated sequentially with amiloride (Am, 10^{-5} M in mucosal bath), forskolin (For, 10^{-5} M in serosal and mucosal baths), methacholine (Mch, 10^{-5} M in both baths), ATP (10^{-5} M in both baths), $CFTR_{inh}$-172 (inh-172, 5×10^{-5} M in mucosal bath), flufenamic acid (FFA, 10^{-4} M in mucosal bath), and ouabain (On, 10^{-4} M in serosal bath). Record is from cells grown on Transwell insert in EGF medium with an air interface.

Table 1. *Electrical properties of cells in EGF medium and 2% USG*

	EGF Medium	2% USG
R_{te}, Ω·cm²	214 ± 34	249 ± 55
I_{sc}, μA/cm²	20 ± 5	26 ± 5
ΔI_{sc}, μA/cm²		
Forskolin	9.5 ± 4.3	14.8 ± 2.8
Methacholine	64 ± 16	85 ± 14
ATP	36 ± 11	42 ± 6

Values are means ± SE; $n = 6$ cell sheets from 3 cultures for 2% Ultroser (USG) and 10 cell sheets from 4 cultures for EGF medium. Cells were grown on Transwell inserts with air-interface feeding and studied at 3–5 days. R_{te}, transepithelial electrical resistance; I_{sc}, short-circuit current.

The responses to methacholine and ATP were not influenced by prior treatment with forskolin. Thus, in the presence of forskolin, the maximal response to methacholine was 126 ± 36 μA/cm² vs. 113 ± 33 μA/cm² in the absence of forskolin (n = 5 tissue pairs from 3 cultures; cells grown on Transwell inserts in EGF medium with an air interface). The response to ATP in the presence of forskolin was 78 ± 12 μA/cm² vs. 78 ± 15 μA/cm² in the absence of forskolin (n = 4 tissue pairs from 3 cultures).

Effects of medium on electrical properties. Table 1 shows that, at 3–5 days, electrical properties of cells grown on Transwell inserts were equivalent in 2% USG and EGF medium. However, electrical properties were significantly lower in cells in EGF-free medium than cells in EGF medium (Table 2). Four pairs of tissues (4–14 days old) from three cultures on Transwell inserts were used for comparison of 5% FCS with 2% USG. Mean values for all the electrical parameters were considerably lower in 5% FCS than 2% USG, although this difference only achieved statistical significance for R_{te} (70 ± 19 vs. 111 ± 18 Ω·cm²) and the I_{sc} response to methacholine (8 ± 3 vs. 81 ± 23 μA/cm²). After stimulation with methacholine, the I_{sc} of cells in 5% FCS returned to baseline within 2 min. In 2% USG or EGF medium, I_{sc} remained considerably above baseline 5 min after addition of methacholine (see below).

Table 2. *Electrical properties of cells in EGF and EGF-free medium*

	EGF Medium	EGF-Free Medium
R_{te}, Ω·cm²	178 ± 28	59 ± 11
I_{sc}, μA/cm²	27 ± 5	14 ± 3
ΔI_{sc}, μA/cm²		
Forskolin	18 ± 3	1.4 ± 0.4
Methacholine	83 ± 11	12 ± 2
ATP	35 ± 6	11 ± 2

Values are means ± SE; $n = 12$ cell sheets from 3 cultures for EGF medium and 11 cell sheets from the same 3 cultures for EGF-free medium. Cells were grown on Transwell or Cyclopore inserts (with the same results) and studied at 3–5 days. All values in EGF-free medium are significantly different from those in EGF medium.

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Protein levels were 261 ± 1 and 181 ± 5 μg/cm^2 in EGF medium and 2% USG, respectively (n = 3). DNA levels were 16.5 ± 0.2 and 14.7 ± 0.2 μg/cm^2 (n = 3) in EGF medium and 2% USG, respectively.

Effects of inserts on electrical properties. In one experiment, cells from the same trachea were grown on four different types of insert immersed in 2% USG. Cells grown on Transwell, Cyclopore, and Millicell-HA inserts attained R_{te} of >100 Ω·cm^2 within ~2 days and were studied in Ussing chambers at 5 days. Cells grown on Transwell inserts attained R_{te} of 166 ± 16 Ω·cm^2, I_{sc} of 19 ± 1 μA/cm^2, maximal increases in I_{sc} to forskolin, methacholine, and ATP of 5 ± 2, 69 ± 9, and 44 ± 2 μA/cm^2, respectively, protein contents of 162 ± 5 μg/cm^2, and DNA contents of 13.0 ± 0.3 μg/cm^2 (n = 3). The electrical properties of cells grown on Cyclopore and Millicell-HA inserts were not statistically different from those of cells grown on Transwell inserts, but protein and DNA levels of cells were significantly less in cells grown on Cyclopore inserts and significantly greater in cells grown on Millicell-HA inserts (Fig. 2). By contrast, cells grown on Millicell-CM inserts failed to develop R_{te} of >100 Ω·cm^2 as determined with the chopstick voltmeter, and in two of three cases, R_{te} was below the limit of detection (~10 Ω·cm^2). In the one tissue with detectable R_{te} and I_{sc}, R_{te}, I_{sc}, and changes in I_{sc} in response to forskolin and methacholine were significantly less than the corresponding mean values for cells grown on the other filter types (Fig. 2).

In another experiment, cells were grown on Cyclopore or Transwell inserts in EGF medium with an air interface. The values (on Transwell and Cyclopore, respectively) for R_{te} (192 ± 36 and 105 ± 29 Ω·cm^2), baseline I_{sc} (28 ± 4 and 32 ± 9 μA/cm^2), maximal I_{sc} increases to forskolin (18 ± 6 and 25 ± 2 μA/cm^2) and methacholine (58 ± 1 and 62 ± 5 μA/cm^2), and protein contents (234 ± 1 and 223 ± 8 μg/cm^2) were not statistically different (n = 3). However, the I_{sc} response to ATP and the DNA contents were significantly less for cells grown on Cyclopore than Transwell inserts: 13 ± 1 vs. 44 ± 1 μA/cm^2 and 11.6 ± 0.1 vs. 14.7 ± 0.2 μg/cm^2.

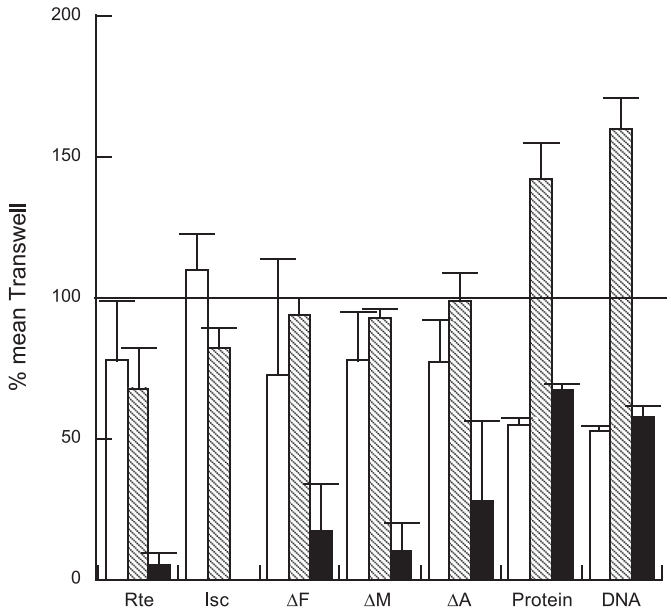

Fig. 2. Effects of insert on electrical properties and protein and DNA content. Values for transepithelial electrical resistance (R_{te}), short-circuit current (I_{sc}), changes in I_{sc} in response to forskolin (ΔF), methacholine (ΔM), and ATP (ΔA), and protein and DNA contents are expressed relative to mean values on Transwell inserts. Open columns, Cyclopore inserts; hatched columns, Millicell-HA inserts; solid columns, Millicell CM inserts. Values are means ± SE, n = 3. Mean values for Transwell inserts are given in RESULTS. Cells were grown immersed in 2% USG.

Immersion vs. air-interface feeding and time dependence of electrical properties. Cells from nine different tracheas were grown in 2% USG on Transwell inserts with an air interface or immersed, and their electrical properties were determined at 2–31 days. Similar results were obtained for the two culture approaches. Figure 3 shows that after 10 days of immersion or air-interface culture, nonviable tissues (R_{te} = 0) started to appear. However, I_{sc} and R_{te} of the remaining viable cells did not show significant alteration (best least-squared linear regressions) over time (Fig. 3). The same was true for the I_{sc} responses to methacholine and forskolin (not shown). The I_{sc} response to ATP of cells grown with an air interface similarly did not change with time. There was, however, a significant decline in the I_{sc} response to ATP over time for immersed cells, with the best least-squares linear regression line predicting a 50% drop in response between 4 and 17 days. When data from viable cells of all ages were pooled, no significant differences in electrical properties were seen between air-interface and immersion feeding (Table 3). Baseline I_{sc} and responses to mediators showed no significant dependence on R_{te} for immersed or air-interface cultures (best least-squared linear regressions).

Time course of responses. When all combinations of USG, EGF, and Transwell, Cyclopore, and Millicell-HA inserts were pooled, the peak response to methacholine was achieved within ~5 s and consisted of an increase in I_{sc} from 23.8 ± 2.4 to 82.0 ± 7.8 μA/cm^2 (n = 46), which declined to 50.4 ± 4.8 μA/cm^2 at 1 min after addition. Thereafter, the decline toward baseline slowed progressively with time. Thus, at 3 min after addition, I_{sc} was 38.6 ± 3.8 μA/cm^2, and at 5 min after addition, it was 33.8 ± 3.2 μA/cm^2, still significantly higher, by 47 ± 7%, than baseline. At this point, ATP was added, so we do not know whether a true sustained response to methacholine would have been achieved. Similar to methacholine, the maximal response to ATP occurred within ~5 s. However, in contrast to methacholine, the response to ATP returned to baseline within ~2 min. There was generally no transient response to forskolin. When there was a transient response (in ~10% of tissues), it was about double the sustained response. The sustained response to forskolin (6.7 ± 1.2 μA/cm^2) was significantly less (paired t-test, n = 44) than the 5-min response to methacholine (11.8 ± 1.2 μA/cm^2).

Effects of transport blockers. Amiloride had no effect on I_{sc} in 48 of 60 tissues (data for all culture conditions combined). When amiloride did have an effect, it was small. Thus, on average, amiloride reduced I_{sc} by 0.6 ± 0.2 μA/cm^2 from a resting level of 19.2 ± 1.6 μA/cm^2 (n = 60).

Forty-one tissues (grown on Cyclopore or Transwell inserts in EGF medium or 2% USG) received FFA at the end of the experiment. At this time, I_{sc} was 30 ± 3 μA/cm^2 (n = 41), and this was inhibited 53 ± 3% by FFA. In a subset of these tissues (n = 27), $CFTR_{inh}$-172 was given before FFA. The baseline I_{sc} of these tissues was 22 ± 3 μA/cm^2, and this was inhibited 10 ± 2% by $CFTR_{inh}$-172 and 60 ± 3% by FFA. Thus the combination of $CFTR_{inh}$-172 and FFA inhibited I_{sc} by 70 ±

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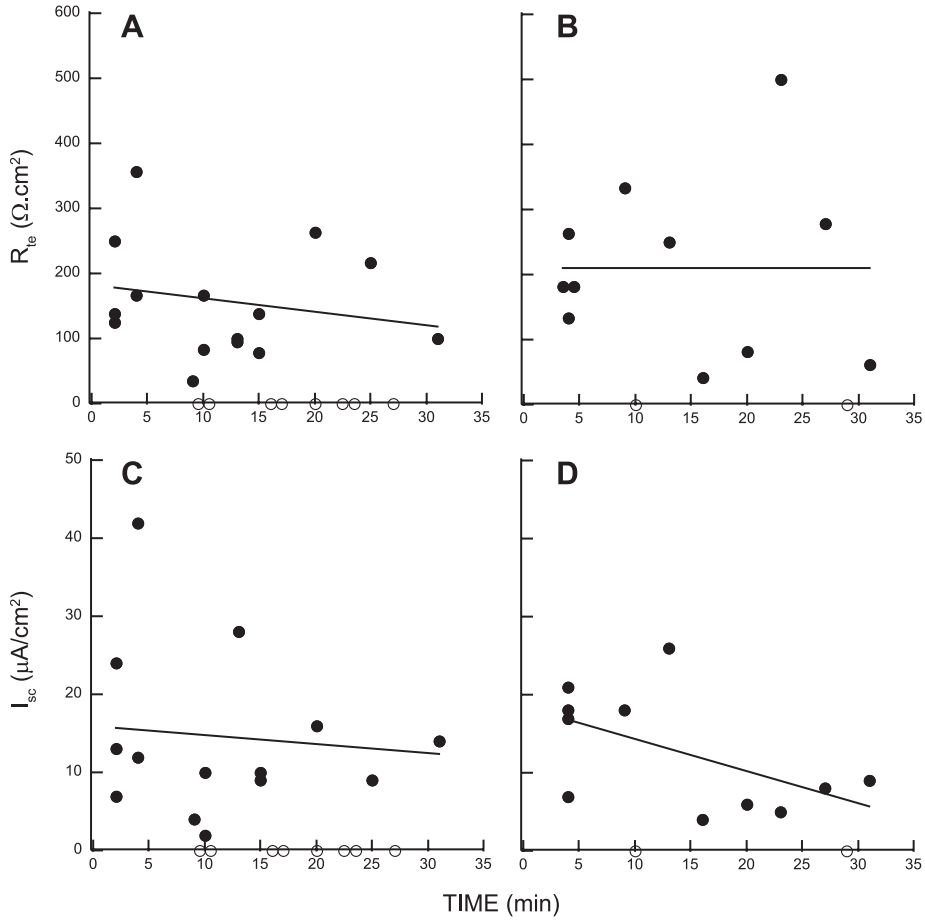

Fig. 3. Time dependence of baseline electrical properties. Cells were grown on Transwell inserts in 2% USG. *A* and *B*: R_{te} with air-interface and immersion feeding, respectively. *C* and *D*: I_{sc} with air-interface and immersion feeding, respectively. ○, Zero values. Regression lines have been drawn through nonzero values (●).

4%. The residual 30% of I_{sc} was abolished by ouabain (Fig. 1). Bumetanide (5×10^{-5} M in the serosal bath) inhibited baseline I_{sc} by 55 ± 7% (n = 9).

Microscopy. Conventional light microscopy and the z stacks of confocal images showed that cell height varied regionally from 10 to 40 μm within individual cell sheets, and the number of cell layers varied from one to four. Cilia were not seen under any culture conditions or at any time in culture.

In our first immunocytochemical studies, we stained individually for lysozyme, lactoferrin, or MUC5B, each of which we estimated to be in ~25% of cells (Fig. 4, *A–C*). A higher proportion of cells stained positively where cell sheets had the most layers and the greatest overall cell height. For all compounds, staining was predominantly in cell apices (Fig. 4, *D–F*).

Table 3. *Electrical properties of cells in air-interface and immersion culture*

	Air-Interface	Immersion
R_{te}, Ω·cm^2	155 ± 21	210 ± 41
I_{sc}, μA/cm^2	14 ± 3	13 ± 2
ΔI_{sc}, μA/cm^2		
Forskolin	6.8 ± 2.4	6.1 ± 1.8
Methacholine	62 ± 14	41 ± 9
ATP	32 ± 8	20 ± 6

Values are means ± SE; n = 17 cell sheets from 7 cultures for air-interface and 11 cell sheets from 3 cultures for immersion feeding; cells sheets from 3 cultures were grown under both conditions. Cells were grown in 2% USG on Transwell inserts.

Later, five cell sheets from three cultures were triple-stained for lactoferrin, lysozyme, and MUC5B. The cells sheets were grown in 2% USG on Transwell inserts and were 4–16 days old. Two had been grown immersed and three with an air-liquid interface. Very similar results were obtained on all five cell sheets, and the results were pooled. There were an average of 255 ± 10 cell profiles per field (n = 60 fields, 12 from each insert). Of these profiles, 13.8 ± 1.9% were positive for lactoferrin, 8.5 ± 0.4% for lysozyme, and 16.9 ± 2.3% for MUC5B. Almost all the cells that stained for lysozyme also stained for lactoferrin; however, a substantial number of cells stained for lactoferrin, but not for lysozyme. Thus, 5.5 ± 1.7% stained for lactoferrin only, 0.2 ± 0.2% for lysozyme only, and 8.3 ± 0.3% for both. The percentage of cells that stained for MUC5B plus one or both of the serous cell markers was 7.6 ± 0.7%, 9.4 ± 2.6% of the cells stained for MUC5B alone, and 6.2 ± 1.4% of the cells stained for one or both of the serous cell markers but *not* MUC5B. Costaining for MUC5AC and MUC5B (on the same cell sheets as used for the triple stain) showed that 27 ± 12% of the cells that stained for MUC5B also stained for MUC5AC. No cells were positive for MUC5AC and negative for MUC5B.

Lysozyme release. PBS was added to the mucosal surface of cells on Transwell inserts grown with an air-liquid interface in EGF medium. A sample, taken immediately after addition, showed 9.0 ± 2.3 units of lysozyme (n = 6) in the PBS (lysozyme was undetectable in PBS that had not been in contact with cells). Whether this lysozyme was present in the

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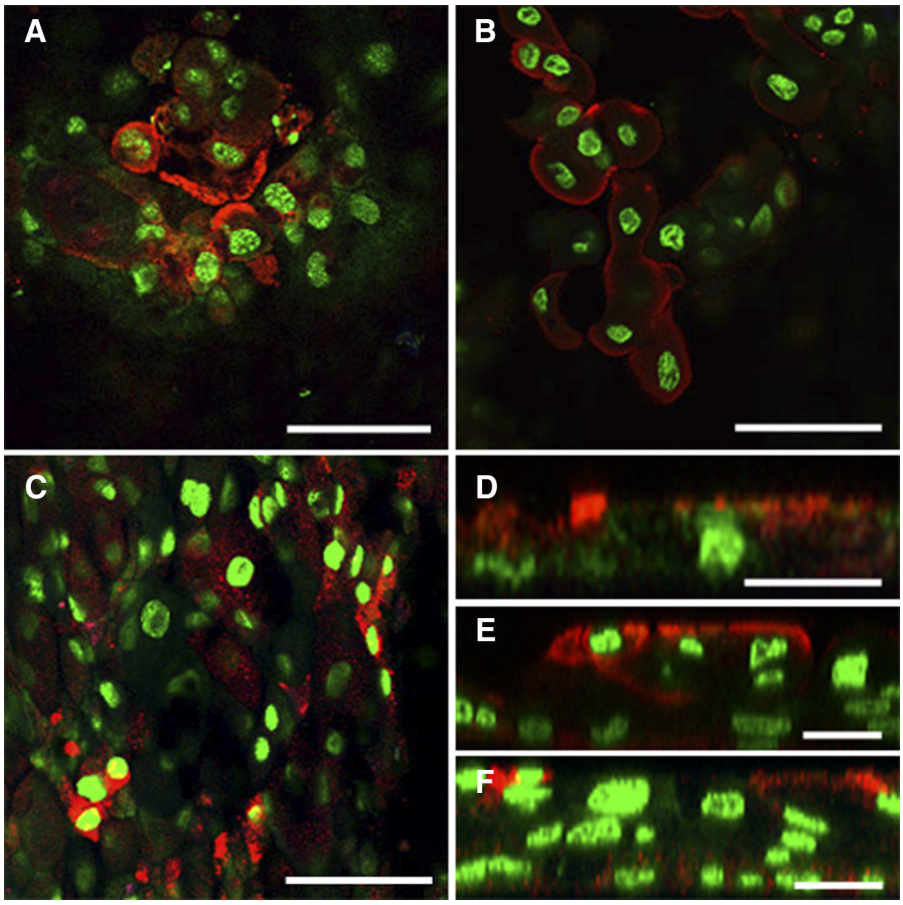

Fig. 4. Confocal fluorescence immunolocalization of gland cell markers: lactoferrin (*A* and *D*), lysozyme (*B* and *E*), and MUC5B (*C* and *F*). *A–C*: confocal sections taken 2–5 μm below the apical membrane. *D–F*: *z* stacks. Positive staining is indicated by red. Nuclei were counterstained with Yo-Pro (green). Scale bars, 50 μm (*A–C*) and 20 μm (*D–F*). Cell sheets were confluent, so absence of nuclei merely means that the apical surface was irregular and disappeared below the focal plane in certain areas.

mucosal liquid or was released from the cells in response to addition of PBS is unclear. Over the next 24 h, a further 5.3 ± 0.6 units of lysozyme were added to the PBS.

Electrical properties and structure of surface epithelial cultures. Surface epithelial cultures were generated from four tracheas grown on Transwell inserts with an air-liquid interface in EGF medium, 2% USG, or 5% FCS and studied in Ussing chambers at 1, 2, 3, or 4 wk (i.e., 12 cell sheets were generated per trachea). The electrical properties and culture requirements of surface epithelial cells were completely different from those of gland cultures. Baseline I_{sc} and the I_{sc} responses to mediators depended on medium in the following sequence: EGF medium >> 5% FCS = 2% USG (Table 4); the sequence for the gland cultures was 2% USG = EGF medium >> 5% FCS. In a comparison of the electrical properties of surface and gland cells grown on Transwell inserts in EGF medium, other important differences stood out. *1*) In the surface cultures, amiloride inhibited baseline I_{sc} by 43 ± 6% (n = 15), as opposed to 3% in the gland cultures (see above). *2*) The maximal responses to forskolin, methacholine, and ATP were approximately equal in surface cultures (Table 4), as opposed to gland cultures, where the sequence was methacholine > ATP >> forskolin (Tables 1 and 3). *3*) The I_{sc} responses of surface cultures to methacholine consisted of step-wise increases (Fig. 5), as opposed to the predominantly transient responses of the gland cultures. In both the gland and surface cultures, FFA was a much more efficacious inhibitor of amiloride-insensitive I_{sc} than was $CFTR_{inh}$-172 (Fig. 5).

After 2 wk, cilia were clearly visible on surface epithelial cells grown on Transwell inserts in EGF medium.

DISCUSSION

We here describe conditions that produce primary cultures of pig tracheal gland epithelium with R_{te} and I_{sc} comparable to those of surface epithelium, both native and cultured (9). Elevation of intracellular Ca^{2+} concentration produced a much

Table 4. *Effects of media on electrical properties and protein and DNA contents of surface epithelial cultures*

	5% FCS	2% USG	EGF Medium
R_{te}, Ω·cm^2	171 ± 35	300 ± 48*	186 ± 15†
I_{sc}, μA/cm^2	8.2 ± 1.3	18.4 ± 1.6*	38.1 ± 4.8*†
ΔI_{sc}, μA/cm^2			
Amiloride	−0.3 ± 0.2	−2.6 ± 0.6*	−16.8 ± 2.8*†
Forskolin	0.5 ± 0.3	0.6 ± 0.5	16.4 ± 2.5*†
Methacholine	1.0 ± 0.3	4.8 ± 1.1*	10.1 ± 1.6*†
ATP	2.7 ± 0.6	4.6 ± 1.0	12.3 ± 2.3*†
Protein, μg/cm^2	193 ± 16	452 ± 43*	304 ± 25*†
DNA, μg/cm^2	3.8 ± 0.5	15.4 ± 1.7*	8.5 ± 1.2*†

Values are means ± SE; n = 16 for 5% FCS and 2% USG and 15 for EGF medium; n = 4 at each of 1, 2, 3, and 4 wk of culture, except for electrical properties at 4 wk in EGF, where n = 3. The only parameter that showed significant change (best least-squared regressions) between 1 and 4 wk was protein content of cells grown in 2% USG, which increased from 356 ± 36 μg/cm^2 at *week 1* to 611 ± 65 μg/cm^2 at *week 4*. *Significantly different from 5% FCS. †Significantly different from 2% USG.

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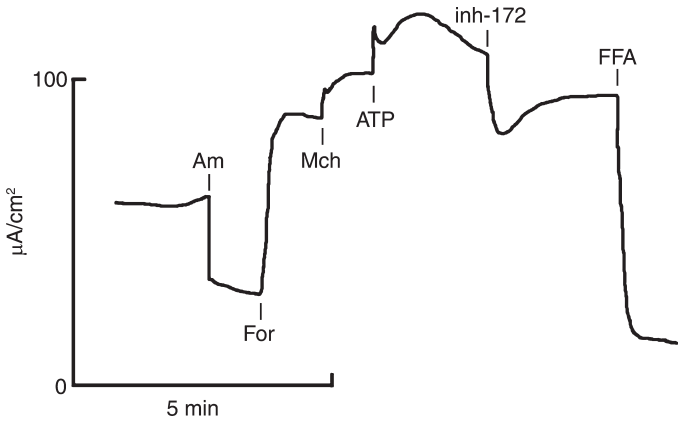

Fig. 5. I_{sc} responses of surface epithelial cell cultures. See Fig. 1 legend for explanation of abbreviations. Record is from a cell sheet grown on a Transwell insert in EGF medium with air-interface feeding.

greater stimulation of anion secretion (amiloride-insensitive I_{sc}) than did elevation of cAMP. Immunocytochemistry showed that the cultures contained MUC5B, the characteristic mucin of airway glands (30), as well as lactoferrin and lysozyme, antimicrobial agents localized specifically to airway gland serous cells (2, 3).

Several culture approaches were taken, with differentiation being initially assessed from R_{te}, baseline I_{sc}, and I_{sc} responses to mediators. We found that 2% USG and EGF medium produced cultures with high values for all these parameters: R_{te} ~200 Ω·cm^2, I_{sc} ~20 μA/cm^2, and maximal I_{sc} responses to forskolin, methacholine, and ATP of ~10, ~80, and ~30 μA/cm^2, respectively. By contrast EGF-free medium produced markedly lower values. Although 5% FCS was not studied in as much detail as the other media, all indications were that it was worse or certainly no better than 2% USG or EGF medium.

In 2% USG or EGF medium, Transwell, Cyclopore, and Millicell-HA inserts produced cells with equivalently high levels of differentiation. By contrast, cells grown on Millicell-CM inserts showed markedly poorer electrical properties, often failing to develop detectable R_{te}. For cells grown on Transwell or Cyclopore inserts in 2% USG, air-interface and immersion feeding produced equivalent electrical properties. After ~10 days, an increasing fraction of cells lost their R_{te}, until at ~30 days, only ~40% of cells had R_{te} greater than zero. Nevertheless, for those cells that retained a measurable R_{te}, there were no time-dependent declines in R_{te}, I_{sc}, or I_{sc} responses to mediators (except for a small decline in the response of immersed cells to ATP).

We prefer Transwell to Cyclopore or Millicell-HA inserts, because they are easier to handle in our Ussing chambers. Also, in EGF medium, cells grown on Transwell inserts give higher responses to ATP than cells grown on Cyclopore inserts. We have found that 2% USG is easier to make than EGF medium. Thus, for routine purposes, we grow cells on Transwell inserts in 2% USG. However, at least six combinations of insert and medium produce cells with similarly high levels of differentiation. Because batches of USG may vary, it is helpful that EGF medium, which is semidefined, produces the same level of differentiation.

The Ca^{2+}-elevating agents methacholine and ATP produced maximal increases in amiloride-insensitive I_{sc} that were five- to eightfold greater than those produced by the cAMP-elevating agent forskolin. The discrepancy between the effects of cAMP and intracellular Ca^{2+} concentration was less (about a 2-fold difference) if the increases in I_{sc} 5 min after addition of methacholine were considered. These results are in excellent agreement with results from intact human and pig glands, most of which show a rapid transient response followed by a slower sustained response to cholinergic agents and sustained secretory rates ~50% greater in response to supramaximal doses of cholinergic agents than to cAMP-dependent agonists (6, 23). Work on human airway gland cell cultures also points to a greater importance of CaCC than CFTR in mediating anion secretion (12). Of course, it is possible for Ca^{2+} elevation to stimulate anion secretion by a CFTR-dependent process; Ca^{2+}-dependent activation of basolateral K^+ channels hyperpolarizes the apical membrane and drives Cl^- (and HCO_3^-) through constitutionally open CFTR. However, this mechanism does not seem important here, because when given at the end of the experiment, FFA (a blocker of CaCC) inhibited amiloride-insensitive I_{sc} nearly threefold more than $CFTR_{inh}$-172, a blocker of CFTR. We acknowledge that none of these anion channel blockers are perfectly selective. However, on the basis of published $K_{0.5}$ values, the FFA concentration used here (100 μM) should produce ~85% inhibition of CaCC (15) and <20% inhibition of CFTR (25). Conversely, the concentration of $CFTR_{inh}$-172 (50 μM) should inhibit CFTR by >95% and CaCC by <5% (24).

Our medium contained 25 mM HCO_3^-, so the amiloride-insensitive I_{sc} presumably reflects a mixture of Cl^- secretion and HCO_3^- secretion. However, the 55% inhibition of baseline I_{sc} by bumetanide suggests that Cl^- secretion is quantitatively more important than HCO_3^- secretion, at least under baseline conditions.

Conventional light microscopy and z stacks from the confocal microscope showed that cells grown on Transwell inserts in USG medium or 2% EGF were 10–40 μm high. Their protein levels were ~250 μg/cm^2. If it is assumed that protein makes up 25% of cell weight (36) and that the extracellular space is 30% of tissue volume, then cell height can be calculated as ~15 μm, in good agreement with the microscopy findings. The apical portions of ~15% of cells grown on Transwell inserts in 2% USG stained positive for lactoferrin, lysozyme, or MUC5B. Positive cells were most frequent and the staining was most intense where the epithelium was highest. In native glands, lactoferrin and lysozyme are localized exclusively to serous cells (2, 3), whereas MUC5B is the predominant mucin of gland mucous cells (30). Staining was much less frequent for MUC5AC than MUC5B. MUC5AC is the predominant mucin of airway goblet cells but is found, although at much lower levels than MUC5B, in mucous gland cells (17). In studies where we costained for MUC5B, lactoferrin, and lysozyme, we found approximately equal numbers of cells that stained for MUC5B alone, for one or both of the serous cell proteins only, or for both MUC5B and lactoferrin or lysozyme. This costaining for mucous and serous cell constituents resembles our initial results from human tracheal gland cultures, in which virtually all cells expressed antigens that stained with antibodies specific to either serous or mucous cells of native glands (33, 40). In future studies, we will attempt to induce the pig tracheal gland cultures to develop more markedly serous or

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mucous phenotypes, as we have recently been able to do with our human tracheal gland cultures (10).

We confirmed that not only was lysozyme detectable in the cells by immunocytochemistry, but it was released into the medium at an average rate of ~5 U/day. Therefore, although of mixed phenotype, the cell cultures developed here should prove useful in studies on the secretion of lysozyme and, possibly, also MUC5B and lactoferrin. We recently showed that, in human cultures, mucin secretion is independent of CFTR-mediated or Ca^{2+}-dependent Cl^- secretion (11). However, in Calu-3 cells, lysozyme secretion is stimulated by cAMP, in parallel with a CFTR-dependent increase in fluid secretion (8, 22). Our cultures could therefore be used to test the hypothesis that lysozyme secretion is (at least in part) CFTR-dependent and is reduced in CF.

In conclusion, we have developed cultures of pig tracheal gland epithelium that form tight junctions and exhibit high R_{te}. In agreement with results from intact human and pig glands and cultures of human glands, we found that CaCC mediates considerably higher levels of anion secretion than CFTR. Pig cultures are much more easily obtained than human cultures and should prove useful in studies on airway gland secretory processes in general. In particular, by comparing results from cultures obtained from wild-type or CF pigs, we can test the hypothesis that secretion of serous cell antimicrobial agents is compromised in CF.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.H.W. and R.M.B. are responsible for conception and design of the research; J.H.W., R.M.B., M.H.-O., M.E.L.-S., W.E.F., J.E.S., and S.M. performed the experiments; J.H.W., R.M.B., M.H.-O., and M.E.L.-S. analyzed the data; J.H.W. interpreted the results of the experiments; J.H.W. prepared the figures; J.H.W., and R.M.B. drafted the manuscript; J.H.W. edited and revised the manuscript; J.H.W., R.M.B., M.H.-O., M.E.L.-S., W.E.F., J.E.S., and S.M. approved the final version of the manuscript.

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