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CFTR and calcium-activated chloride channels in primary cultures of human airway gland cells of serous or mucous phenotype

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Fischer H, Illek B, Sachs L, Finkbeiner WE, Widdicombe JH. CFTR and calcium-activated chloride channels in primary cultures of human airway gland cells of serous or mucous phenotype. *Am J Physiol Lung Cell Mol Physiol* 299: L585–L594, 2010. First published July 30, 2010; doi:10.1152/ajplung.00421.2009.—Using cell culture models, we have investigated the relative importance of cystic fibrosis transmembrane conductance regulator (CFTR) and calcium-activated chloride channels (CaCC) in Cl secretion by mucous and serous cells of human airway glands. In transepithelial recordings in Ussing chambers, the CFTR inhibitor $CFTR_{inh}$-172 abolished 60% of baseline Cl secretion in serous cells and 70% in mucous. Flufenamic acid (FFA), an inhibitor of CaCC, reduced baseline Cl secretion by ~20% in both cell types. Methacholine and ATP stimulated Cl secretion in both cell types, which was largely blocked by treatment with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) and partially by mucosal FFA or $CFTR_{inh}$-172 with the exception of methacholine responses in mucous cells, which were not blocked by FFA and partially (~60%) by $CFTR_{inh}$-172. The effects of ionomycin on short-circuit current (I_{sc}) were less than those of ATP or methacholine. Forskolin stimulated Cl secretion only if Cl in the mucosal medium was replaced by gluconate. In whole cell patch-clamp studies of single isolated cells, cAMP-induced Cl currents were ~3-fold greater in serous than mucous cells. Ionomycin-induced Cl currents were 13 times (serous) or 26 times (mucous) greater than those generated by cAMP and were blocked by FFA. In serous cells, mRNA for transmembrane protein 16A (TMEM16A) was ~10 times more abundant than mRNA for CFTR. In mucous cells it was ~100 times more abundant. We conclude: *1*) serous and mucous cells both make significant contributions to gland fluid secretion; *2*) baseline Cl secretion in both cell types is mediated predominantly by CFTR, but CaCC becomes increasingly important after mediator-induced elevations of intracellular Ca; and *3*) the high CaCC currents seen in patch-clamp studies and the high TMEM16A expression in intact polarized cells sheets are not reflected in transepithelial current recordings.

airway mucous cells; airway serous cells; TMEM16A; whole cell patch-clamp

SEVERAL LINES OF EVIDENCE suggest that most of the liquid secreted by human airway submucosal glands is dependent on cystic fibrosis (CF) transmembrane conductance regulator (CFTR). First, serous cells are believed to provide most of the liquid in gland secretions (36), and airway CFTR has been reported as being localized predominantly to the serous cells in gland acini (10). Second, in primary cultures of epithelium from human airway glands, we found that short-circuit current (I_{sc}) responses to a variety of neurohumoral mediators were essentially abolished in CF (56). Third, diphenylamine-2-carboxylate (DPC) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) block gland secretion induced by either forskolin or methacholine in pig airways (2), but DIDS has no effect. This blocker selectivity is more consistent with CFTR than calcium-activated chloride channels (CaCC) (16, 17, 19). Fourth, $CFTR_{inh}$-172, a blocker of CFTR (33), inhibited cholinergically induced airway gland secretion in pigs and in humans without CF but not in humans with CF (48). Fifth, gland secretion rate in CF nasal biopsies is ~40% of normal (42). Finally, CFTR is the major apical membrane Cl channel of Calu-3 cells (40, 45), a cell line often assumed to be a good model for gland serous cells.

Much of this evidence can be questioned. For instance, the most recent immunohistochemical studies suggest that CFTR is localized predominantly in ciliated cells of the surface epithelium and is rarely found in the acini of submucosal glands (29). The Cl channel blockers used in the studies of Ballard et al. (2) on pig airway gland secretion are not specific for CFTR (17). Choi and colleagues (7) have recently reported that maximal rates of secretion in response to cholinergic agents are not altered in CF. In fact, the earlier reports of a difference (42) could have been due to unintentional investigator bias. Thus the nasal biopsies used were stated to be ~4 mm in size, so they would be ~16 mm^2 if square or ~13 mm^2 if circular. Given 1 gland per mm^2 (6, 49, 50), there would have been ~15 glands per biopsy, yet secretions from only 3 to 6 glands per biopsy were measured, and no information was given as to how or why this subset of glands was selected. This is especially troubling given that individual glands vary by >10-fold in their baseline secretory rates and in their secretory responses to mediators (25–27). In our initial studies of I_{sc} across cultures of human airway glands (56), the transepithelial electrical resistance (R_{te}) of the CF cells (171 $\Omega \cdot cm^2$) was significantly less than that of the non-CF (541 $\Omega \cdot cm^2$). As there is no evidence for a change in R_{te} of native airway epithelium in CF (4), it is possible that the differences we saw between CF and non-CF cells were caused to some degree by culture-related phenomena (e.g., differences in the composition of the initial cell suspensions, in the plating efficiencies, or in the rates of growth). In later studies, we found the I_{sc} responses of CF cells to mediators were not abolished but reduced by 50–80% depending on mediator (51). Finally, the frequently used Calu-3 cell model for serous gland cells (31) has limitations. Calu-3 cells are derived from an adenocarcinoma of the lung (15), and when grown as air liquid interface cultures show scattered cells with mucous granules (30, 45). Calu-3 cells do not secrete lactoferrin, a major secretory product of serous cells, and their rate of lysozyme secretion is also probably much less than for native serous cells (9).

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By contrast to the increasing confusion surrounding the role of CFTR in gland function, evidence for a role of CaCC has been increasing. For instance, the secretory response of non-CF glands to calcium-elevating agents is much greater than to cAMP-dependent secretagogues (27). This, of course, does not eliminate the possibility that Ca may activate basolateral K channels, hyperpolarize the cells, and drive a net flux of Cl through CFTR that is constitutively open. However, CF airway glands also show robust secretory responses to methacholine (7, 25), an agent that not only acts via elevation of intracellular Ca, but also is the main physiological gland secretagogue (12). By contrast, the response to cAMP-elevating agents (forskolin and VIP) is abolished in CF (25). Patch-clamp studies on isolated human airway gland cells show that CaCC account for the increases in Cl current induced by cholinergic agents (43, 44, 52). In isolated serous gland cells from the mouse, Ca elevation caused by cholinergic or other agents resulted in loss of intracellular Cl and shrinkage (32). cAMP-elevating agents, however, were without detectable effect on cell volume, and responses to cAMP of cells from mice lacking CFTR were the same as wild-type cells (32).

To help determine the relative importance of CFTR and CaCC to human airway gland secretion, we have grown confluent sheets of human primary serous and mucous cells on porous inserts and measured their Cl secretory responses as I_{sc} in Ussing chambers. We then compared these results with the whole cell patch-clamp currents of freshly isolated cells. Finally, as there is now compelling evidence that the molecular identity of CaCC is the protein TMEM16A (5, 57), we compared mRNA for TMEM16A and CFTR in mucous and serous cells.

MATERIALS AND METHODS

Cultures of human tracheal gland cells were initiated as previously described (46). Research protocols were approved by the Institutional Review Board of the University of California, San Francisco. In brief, acini were obtained by enzymatic dispersion and plated into T-25 flasks. Cells grew out from the attached acini, and when 80% confluent they were trypsinized. Plating of cells onto Transwells (Cole-Parmer, Vernon Hills, IL) in hormonally defined medium with EGF produced mucous cells, whereas growth on Cyclopore filters (Whatman, Piscataway, NJ) in medium lacking EGF produced serous cells (13). All inserts were coated with human placental collagen as previously described (8). Human surface airway epithelial cells, Calu-3 cells, and CFBE41o$^-$ cells stably expressing recombinant CFTR (termed here CFBE+CFTR cells) were cultured exactly as in previous studies (20, 22, 41).

R_{te} and transepithelial electrical voltage (V_{te}) were checked every 2 days with a "chopstick" voltmeter (Millicell-ERS; Millicell). When R_{te} was $\geq$100 $\Omega \cdot cm^2$, filters were removed from the bottom of the inserts and studied in conventional Ussing chambers as previously described (55). Cell sheets were studied from 10 to 16 days after plating, and we could discern no dependence of the electrical properties of either serous or mucous cultures with age over this period. Nevertheless, both phenotypes derived from any given trachea were studied on the same day or days after plating. Thus the average ages of both phenotypes studied were identical.

I_{sc} measurements were made at 37°C in conventional Ussing chambers of 0.6 cm^2 (22) or 1 cm^2 (55) connected to gas-lift oxygenators, with I_{sc} being continuously recorded on a computer or pen recorder. Every 20 s, voltage pulses of 0.5 or 1 mV were applied for 0.2 or 2 s to determine R_{te}. Experiments were done with symmetric Cl, or with a serosal-to-mucosal Cl gradient. The chloride-containing solution was 120 mM NaCl, 20 mM $NaHCO_3$, 5 mM $KHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 5.6 mM glucose. In the chloride-free solution, all Cl salts were exchanged for gluconate salts, and Ca was increased to 5 mM to compensate for the chelation of Ca by gluconate. Both chamber compartments were gassed with 95% O_2-5% CO_2 (pH 7.4). Experiments were done at 37°C. After amiloride was added to inhibit active Na^+ absorption, a series of agonists and antagonists of Cl secretion were given. All additions were cumulative; agents were not washed out.

For patch-clamp experiments, mucous, serous, and, for comparison, surface epithelial cells were trypsinized from filters (5-min trypsin in calcium-free PBS added to the mucosal surface), reseeded on Cell-Tak-coated glass coverslips (BD Bioscience, San Jose, CA), and used after 1–6 h. Cells were investigated in the whole cell patch-clamp mode as previously described (14). Briefly, recordings were done with symmetric *N*-methyl-D-glucamine chloride (NMDG-Cl) solutions, and the membrane potential (V_m) was clamped to −50 mV and pulsed to 0 mV to monitor the membrane conductance. Bath solution contained 150 mM NMDG, 145 mM HCl, 1.7 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 10 mM sucrose, pH 7.3. The pipette solution contained 150 mM NMDG, 145 mM HCl, 2 mM EGTA, 1 mM $MgCl_2$, 2 mM HEPES, 1 mM glucose, 5 mM MgATP, pH 7.3, adjusted with NMDG. To stimulate CFTR, 0.5 mM Na-cAMP was included in the pipette. Cell membrane capacitance (C_m) and access resistance (R_a) were estimated from the current transient induced by a 5-mV voltage pulse. Whole cell conductance (G_m) was calculated from currents as the slope conductance measured between −50 and 0 mV, and the specific membrane conductance was normalized to the membrane capacitance (G_s in picosiemens per picofarad).

For measurement of mRNA for CFTR and TMEM16A, cell cultures were lysed in RLT buffer (Qiagen, Valencia, CA) with 1% 2-mercaptoethanol and 0.4% linear acrylamide and stored at −80°C. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) along with a 30-min on-column DNase digestion. Total RNA was quantified on a NanoDrop ND-1000 spectrometer and normalized to 20 ng/μl in nuclease-free water, and 100 ng of total RNA was used for subsequent cDNA reactions. cDNAs were prepared using a two-step protocol. In *step 1*, final concentrations were 100 ng of RNA, 5 μM random decamers (Ambion, Foster City, CA), and 5 mM each dNTP. This mixture was heated to 65°C for 3 min and 5 min at 25°C in an Applied Biosystems (Foster City, CA) model 2720 thermocycler. The reaction was then cooled on ice. For the second reaction, 100 units of SuperScript III (Invitrogen, Carlsbad, CA) 1× reaction buffer, 10 mM dithiothreitol (DTT), and 20 units of RiboLock (Fermentas, Glen Burnie, MD) were added to the RNA mixture and heated for 25°C for 10 min, 42°C for 60 min, and 95°C for 2 min. PCR reactions were performed with *Taq*Man PCR primers and probes provided by Applied Biosystems for CFTR (Hs00357011_m1), TMEM16A (Hs00216121_m1), and 18S rRNA (4319413E). Real-time PCR assays were carried out in triplicates of 15-μl reaction volumes. The reactions for both TMEM16A and CFTR were multiplexed with the 18S rRNA. Real-time PCR was performed on an Applied Biosystems AB7900 Sequence Detection System running SDS 2.1 software. The PCR product sizes were expected to be 75 bp (TMEM16A), 93 bp (CFTR), and 187 bp (18S rRNA). This was verified using ethidium bromide staining of standard 1.5% agarose gels.

Analysis of mRNA expression was performed compared with 18S rRNA using the difference of PCR cycles to reach a threshold amplification (ΔC_T), and the relative amount of the target mRNA is given as $2^{-\Delta CT}$. To verify comparable primer binding, standard curves were generated for both target and endogenous control genes using serial dilutions of cDNA using 0.4 μM forward and reverse primers and 50 ng of cDNA. To control for specific PCR products, a dissociation curve was generated after the end of the last cycle.

Most pharmacological agents were obtained from Sigma (St. Louis, MO) and added as 1:100 or 1:1,000 dilutions of stocks made up in

water, ethanol, or DMSO. Addition of vehicle was without effect on any of the properties measured.

Data are presented as means ± SE; *n* refers to the number of cultures or cells investigated. Comparisons of groups were performed using *t*-tests; $P < 0.05$ was considered statistically significant.

RESULTS

Transepithelial characteristics in symmetric chloride. In symmetric NaCl solutions, both serous and mucous cultures were moderately tight (Table 1), and mucous cultures showed significantly higher basal I_{sc} than serous cultures (Fig. 1*A*, Table 1). Amiloride (50 μM, mucosal) reduced I_{sc} by 23 ± 7% in serous cells and 12 ± 3% in mucous cells. Forskolin (20 μM, serosal) had insignificant effects on I_{sc} in both culture types (Table 1). However, the Ca agonists, methacholine and ATP (100 μM, mucosal and serosal), stimulated currents in both types of cells, with larger responses in mucous cultures (Table 1). The stimulations were generally transient in nature, but in some cases methacholine resulted in sustained increases in I_{sc}. Ionomycin (10^{-6} M on both sides of the tissue) had effects on I_{sc} that were less than those for either ATP or methacholine (Table 1). $CFTR_{inh}$-172 (20 μM, mucosal), a blocker of CFTR (33), added at the end of the experiment reduced I_{sc} by 57 ± 6% (serous) or 70 ± 2% (mucous).

Furthermore, we investigated the effects of the Ca chelator 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), the CaCC blocker flufenamic acid (FFA), and $CFTR_{inh}$-172 on the current responses to ATP and methacholine in mucous and serous cells. Two example recordings of the effect of FFA in mucous cells are shown in Fig. 1*B*, and the summary of all drug effects in both cell types is shown in Fig. 1, *C–F*. When cells were exposed to BAPTA-AM (50 μM, 5 min), the responses to ATP and methacholine were significantly reduced in both cell types, indicating that intracellular Ca mediated the measured current responses in both cell types.

FFA (100 μM, mucosal surface) was used to determine whether CaCC played a role in baseline or mediator-induced I_{sc}. This agent inhibits CaCC and TMEM16A with IC_{50}s of ~50 μM (5, 17). By contrast, its IC_{50} for inhibition of CFTR is ~1 mM (35). Cells were treated sequentially with amiloride, FFA, methacholine, and ATP. Control cells received amiloride, methacholine, and ATP before addition of FFA. Representative records are shown in Fig. 1*B*. After addition of amiloride, the I_{sc}s of control and test cultures were not statistically different (9.2 ± 1.7 and 10.2 ± 2.0 μA/cm², respectively; $n = 4$ for both groups). Added before methacholine or ATP, FFA inhibited agonist responses in serous cultures (Fig. 1, *C* and *D*), suggesting a role for CaCC. In mucous cultures, FFA inhibited the responses to ATP but not to methacholine (Fig. 1, *E* and *F*). An example tracing for this behavior is shown in Fig. 1*B*, *bottom*. The simplest explanation of this observation is that in mucous cells, the ATP-response is mediated by CaCC, whereas the methacholine response is mediated by activation of basolateral K channels, however, this has not been further tested here.

Table 1. *Electrical properties of serous vs. mucous cell cultures*

	Symmetric		Cl Gradient	
	Serous	Mucous	Serous	Mucous
R_{te}, Ω·cm²	263 ± 21	368 ± 48	305 ± 79	403 ± 182
I_{sc}, μA/cm²	15 ± 2	35 ± 5*	22.6 ± 5.3	45.5 ± 23
ΔI_{sc} (For)	0.0 ± 0.1	0.9 ± 0.6	4.8 ± 2.2	11.0 ± 4.5
ΔI_{sc} (MCh)	2.6 ± 0.6	14.4 ± 3.5*		
ΔI_{sc} (ATP)	2.9 ± 1.6	4.9 ± 2.2	4.4 ± 3.3	17.5 ± 2.1*
ΔI_{sc} (ionomycin)	2.0 ± 0.7	−1.4 ± 0.1		

Values are means ± SE, $n = 6–8$ for symmetric conditions, and $n = 3–5$ for Cl gradient conditions. *Statistically significantly different between cell types, $P < 0.05$. R_{te}, transepithelial electrical resistance; I_{sc}, short-circuit current; For, forskolin; MCh, methacholine. All current responses were measured in presence of mucosal amiloride (50 μM).

For comparison, serosal FFA had no effect on I_{sc} of amiloride-pretreated mucous or serous tissues ($n = 3$), nor did serosal FFA affect the response to methacholine (8.1 ± 1.1 μA/cm² with FFA vs. 6.0 ± 0.8, $n = 4$ without); however, the response to ATP in the presence of FFA (7.9 ± 1.0 μA/cm²) was significantly less than control (13.8 ± 1.1 μA/cm², $n = 4$).

We investigated the effects of mucosal $CFTR_{inh}$-172 on mediator-induced responses. Given immediately after amiloride and before addition of the agents, $CFTR_{inh}$-172 reduced I_{sc} by 54 ± 4% ($n = 8$). Given after amiloride and the mediators, it inhibited I_{sc} by 58 ± 7% ($n = 8$). $CFTR_{inh}$-172 significantly reduced the responses to ATP and methacholine in both mucous and serous cells (Fig. 1, *C–F*).

Transepithelial characteristics with a serosal-to-mucosal chloride gradient. The inhibition of I_{sc} by $CFTR_{inh}$-172 in symmetric bathing media indicated a role for CFTR in Cl secretion across both serous and mucous cells. So the lack of effect of forskolin on I_{sc} was surprising. One possible explanation for this negative result was that a stimulatory effect of opening CFTR might have been offset by a resulting depolarization. To test this hypothesis, Ussing chamber experiments were done using mucosal chloride-free solutions (Cl replaced by gluconate). Under these circumstances, a depolarization induced by opening of CFTR should have a comparably small effect on the net electrochemical driving force for Cl. In the presence of amiloride, forskolin significantly increased I_{sc} by both types of cultures (Fig. 2 and Table 1). Added after forskolin, the K channel activator, 1-ethyl-2-benzimidazolinone (EBIO; 1 mM in the serosal bath) increased currents by a further 2.0 ± 1.2 μA/cm² in serous cells and 5.5 ± 0.2 μA/cm² in mucous cells, suggesting that transepithelial recordings of CFTR activity with symmetric chloride were limited by a lack of a driving force. Forskolin and EBIO-stimulated currents were largely blocked by the CFTR inhibitor GlyH-101 (38) at 20 μM in the mucosal bath: serous, −17.4 ± 5.0 μA/cm²; mucous, −5.6 ± 2.4 μA/cm². In the presence of GlyH-101, mucosally added ATP (100 μM) stimulated significantly larger currents in mucous compared with serous cultures (Fig. 2, Table 1), suggesting a larger role of CaCC in mucous compared with serous cells.

Whole cell patch-clamp studies. Interpretation of the Ussing chamber studies described above was made difficult by lack of information on the electrochemical driving force for Cl across the apical membrane. To better control the concentration gradients and voltages across the apical membrane, we attempted to permeabilize the basolateral membranes using α-toxin, nystatin, or amphotericin. However, none of these approaches resulted in a reliable permeabilization (data not shown), as assessed by standard criteria (14, 23). Therefore, to test further for the relative contributions of CaCC and CFTR to the plasma membrane Cl conductance of serous and mucous cells, we

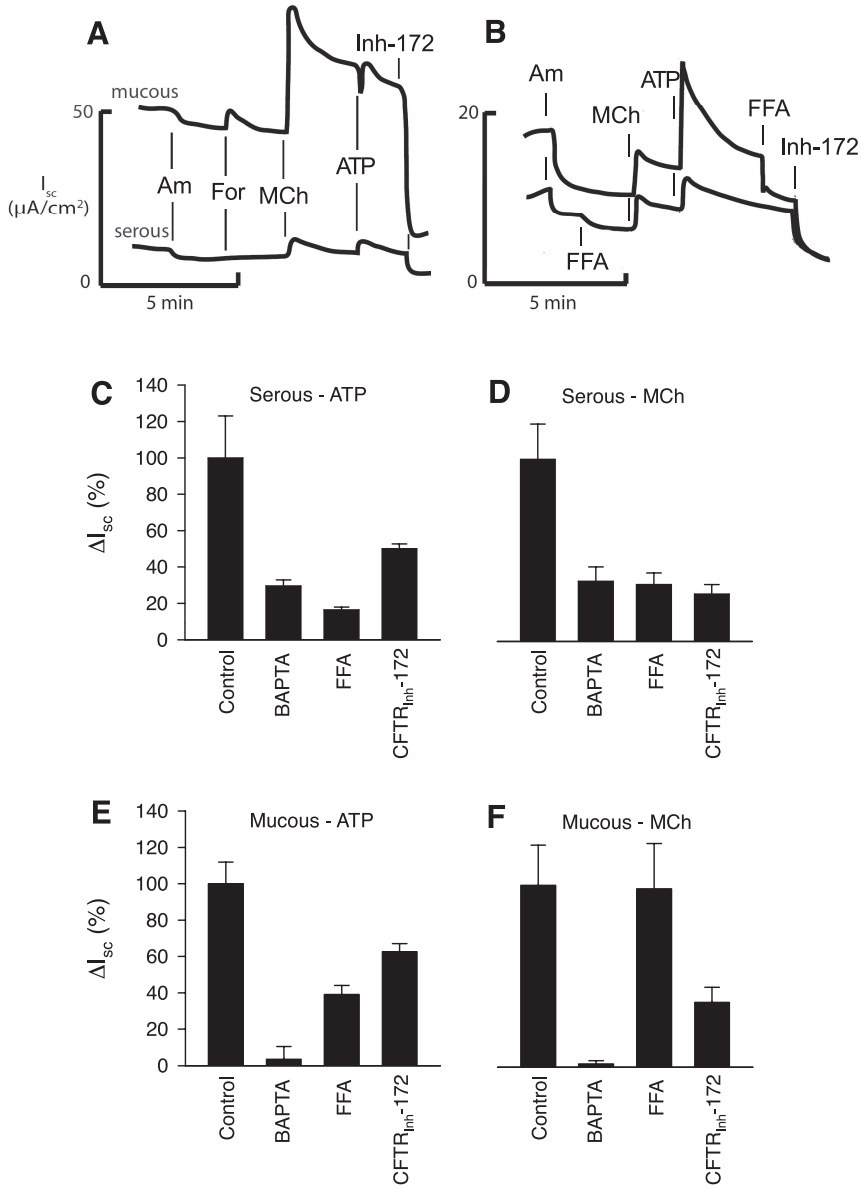

Fig. 1. Short-circuit current (I_{sc}) records in symmetric Cl media. *A*: representative recordings from mucous and serous cultures derived from the same trachea. *B*: effects of flufenamic acid on mucous cells. Both tracings are from mucous cell cultures; differences in baseline currents reflect the variations seen in this study. Am, 50 μM amiloride, mucosal; For, 20 μM forskolin, serosal; MCh, 100 μM methacholine, mucosal and serosal; Inh-172, 20 μM cystic fibrosis transmembrane conductance regulator inhibitor $CFTR_{inh}$-172, mucosal; FFA, 100 μM flufenamic acid, mucosal. *C*: current responses to ATP in serous cells. Control current response was 7.6 ± 1.75 μA/cm^2. BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. *D*: current responses to MCh in serous cells. Control current response was 2.15 ± 0.42 μA/cm^2. *E*: current responses to ATP in mucous cells. Control current response was 8.4 ± 1.3 μA/cm^2. *F*: current responses to MCh in mucous cells. Control current response was 5.9 ± 1.3 μA/cm^2; $n = 4$ for each bar.

performed whole cell patch-clamp studies. Cells were trypsinized from filters, reseeded on Cell-Tak-coated glass coverslips, and used within 6 h of isolation. Visually, the cells maintained their phenotype during this procedure with serous cells containing small granules (Fig. 3*A*) and mucous cells displaying large granules, some of which could be seen in the process of discharge (Fig. 3*B*).

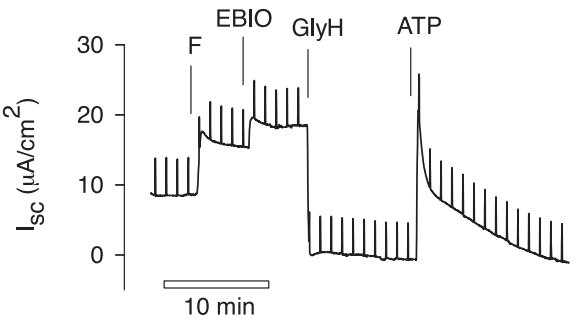

Fig. 2. Representative I_{sc} record for mucous cells in asymmetric Cl media. Recording was done in presence of mucosal amiloride (50 μM). F, forskolin; EBIO, 1-ethyl-2-benzimidazolinone; GlyH, CFTR inhibitor GlyH-101.

In initial recordings, we attempted to stimulate cells with forskolin in the whole cell mode. However, responses were inconsistent, possibly owing to the uncontrolled dialysis of cellular cAMP in the whole cell patch-clamp mode. Therefore, 0.5 mM cAMP was included in the patch pipette; this concentration can be expected to maximally stimulate CFTR (21). Figure 3, *A* and *B*, shows representative recordings from serous and mucous cells, respectively. The *bottom* traces in Fig. 3 show the applied holding potential, which was clamped to a baseline of −50 mV and pulsed to 0 mV, and current-voltage step protocols were applied as shown. Typical current responses to voltage steps are shown in detail in Fig. 4. Whole cell currents measured in presence of cAMP were generally small. The corresponding specific Cl conductances were significantly larger in serous than in

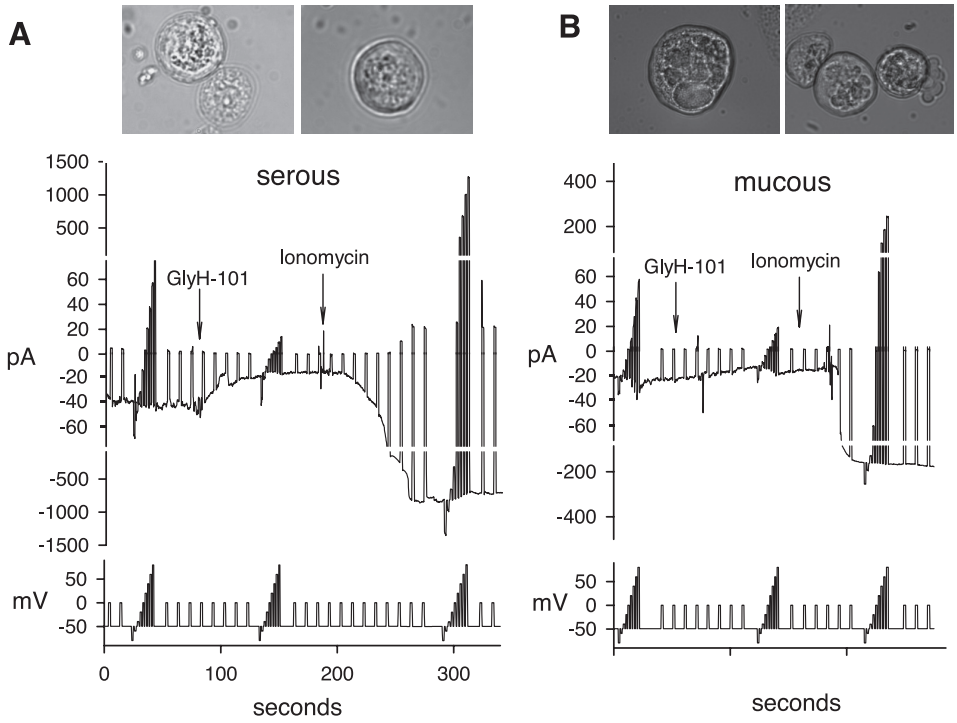

Fig. 3. Whole cell patch-clamp recordings from isolated serous and mucous cells. *A*: serous cells. *B*: mucous cells. *Top* shows images of cells as they appeared after isolation for patch-clamping; magnification, ×600. *Middle* shows current traces; note breaks in *y*-axis scaling. All drug additions were additive: 20 μM GlyH-101, 1 μM ionomycin, and pipette contained 0.5 mM cAMP. *Bottom* shows the applied voltages; baseline = −50 mV, pulsed to 0 mV, voltage steps from −80 to 80 mV (step = 20 mV).

mucous cells (Table 2); the latter showed a similar specific conductance as airway surface cells (Table 2). In all cell types, GlyH-101 blocked considerable fractions of currents (serous, 66 ± 3.7%, n = 4; mucous, 67 ± 4.4%, n = 3; surface, 53 ± 16%, n = 3). The current-voltage relations (Fig. 4, *A–C*) in the presence of cAMP were time- and voltage-independent, and GlyH-101 showed a voltage-dependent block (38) such that negative currents were less well-blocked, resulting in an inwardly rectifying current after GlyH-101 (Fig. 4, *B* and *C*). These are typical characteristics of CFTR-mediated currents and were not different between cell types (data not shown).

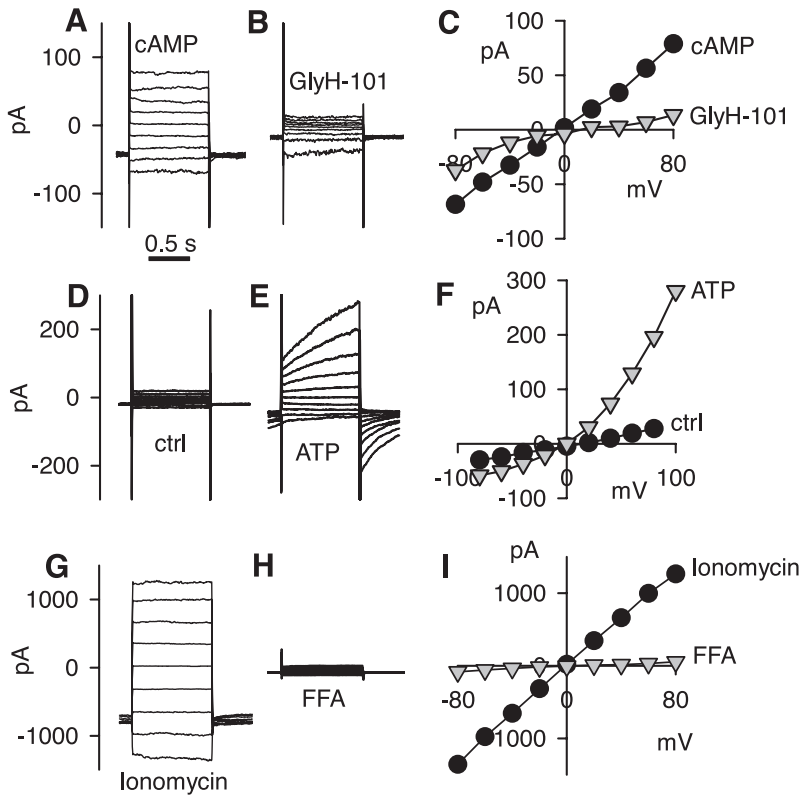

Fig. 4. Currents evoked by voltage steps and current voltage plots. Holding potential was stepped from −80 mV to +80 mV in 20-mV increments. *A–C*: recordings from a serous cell in presence of cAMP. *A*: currents with cAMP. *B*: 20 μM GlyH-101 causes voltage-dependent block. *C*: current-voltage plots before and after addition of GlyH-101. *D–F*: recordings from a mucous cell without cAMP. *D*: control (ctrl) baseline currents. *E*: currents in presence of 100 μM ATP in the bath. *F*: current voltage plots before and after addition of ATP. *G–I*: ionomycin-stimulated currents recorded from a mucous cell. *G*: large currents in presence of 1 μM ionomycin. *H*: block of current by 100 μM FFA. *I*: current-voltage plots before and after addition of FFA.

Table 2. *Specific membrane conductance of serous, mucous, and surface airway cells in whole cell patch-clamp recordings*

			Specific Conductance, pS/pF	
	n	C_m, pF	cAMP	Ionomycin
Serous	6	20 ± 4.1	50.6 ± 12.7*	651 ± 73†
Mucous	8	21 ± 3.8	16.3 ± 4.1	416 ± 152
Surface	4	17 ± 1.5	14.4 ± 5.5	176 ± 75

Values are means ± SE. cAMP (0.5 mM) was present in the intracellular pipette solution; 1 μM ionomycin was added to the bath. *Significantly different from other cell types; †significantly greater than surface epithelial cells (by ANOVA). C_m, cell membrane capacitance.

Currents mediated by CaCC were measured in the presence of GlyH-101 to block CFTR. Initially, we attempted to activate currents by adding ATP to the bath (100 μM). However, responses were small and inconsistent (2 responders in 6 trials). An ATP-activated current is shown in Fig. 4, *D–F*, which shows a slowly depolarization-activated, outwardly rectifying current that is typical for CaCC-mediated currents at low intracellular Ca concentrations ($[Ca]_i$; 11). To maximally stimulate CaCC, we used ionomycin (1 μM). Addition of ionomycin to the bath stimulated very large Cl currents of similar magnitude in both cell types (Fig. 3, *A* and *B*). These currents were time- and voltage-independent (Fig. 4*G*), which is typical of CaCC when recorded at maximal Ca concentrations (11). Ionomycin-stimulated currents were fully blocked by FFA (100 μM; Fig. 4, *H* and *I*). Compared with CFTR-mediated currents, CaCC conductances were 13 times larger in serous and 26 times larger in mucous cells.

Determination of CFTR and TMEM16A expression. Because it seems highly probable that the bulk of calcium-activated Cl currents in airway surface epithelium (5) and other tissues (18) are mediated by TMEM16A, we compared levels of mRNA for this protein and for CFTR in serous and mucous gland cell cultures. Cells were cultured to confluency, mRNA was isolated and transcribed into cDNA, and CFTR and TMEM16A expression were determined by real-time PCR using *Taq*Man probes and primers. Results were normalized to 18S rRNA. For comparison, we included 2 cell lines in this analysis: Calu-3 are known to express large CFTR currents, and CFBE41o$^-$ cells express CFTR (recombinantly) and CaCC (20, 22). Figure 5*A* shows that we were able to obtain transcripts of the predicted size in a control PCR gel. Figure 5*B* shows the relative levels of transcripts for CFTR and TMEM16A. Serous cells expressed ~2.2 times higher levels of CFTR mRNA than mucous cells consistent with the observation of higher CFTR-mediated membrane conductance (~3 times; Table 2). Both expressed less CFTR mRNA than the Calu-3 and CFBE+CFTR cell lines, which are known to express high levels of CFTR (Fig. 5*B*). TMEM16A was found to be least expressed in Calu-3 cells (Fig. 5), which were previously shown to express only small CaCC currents (20). Serous cells expressed 16 times and mucous cells 72 times the amount of TMEM16A mRNA as Calu-3 cells. Thus mucous cells had considerably more TMEM16A transcripts than did serous. The expression of TMEM16A mRNA appeared considerably larger than CFTR mRNA in both serous (12 times) and mucous (117 times) cells.

DISCUSSION

We have compared levels of cAMP and calcium-mediated Cl secretion in primary cultures of human tracheal glands that resemble either serous or mucous cells of native tissue. Several results on serous and mucous cells were qualitatively the same. When the cultures were in the form of intact polarized cell sheets, both cell types actively secreted Cl, and it appeared that about two-thirds of basal Cl secretion was carried by CFTR. Use of specific blockers for CFTR or CaCC indicated that Cl secretory responses to the calcium-elevating agent, methacholine, were mainly CFTR-dependent (presumably reflecting activation of K channels, hyperpolarization, and increased driving force for Cl exit through constitutively open CFTR). By contrast, ATP appeared to act approximately equally via activation of CaCC and via increased driving force through CFTR. In whole cell patch-clamp recordings of isolated cells, ATP activated calcium-dependent currents that were of similar magnitude to those elicited by cAMP. By contrast, ionomycin-induced calcium-activated Cl currents were much greater than the cAMP-activated currents. However, ionomycin had trivial effects on Cl secretion across polarized cell sheets. In both serous and mucous cells, levels of mRNA for TMEM16A were an order of magnitude greater than the levels of mRNA for CFTR.

Baseline I_{sc} was twofold greater in mucous than serous cells, a difference we (13) have elsewhere ascribed to the greater

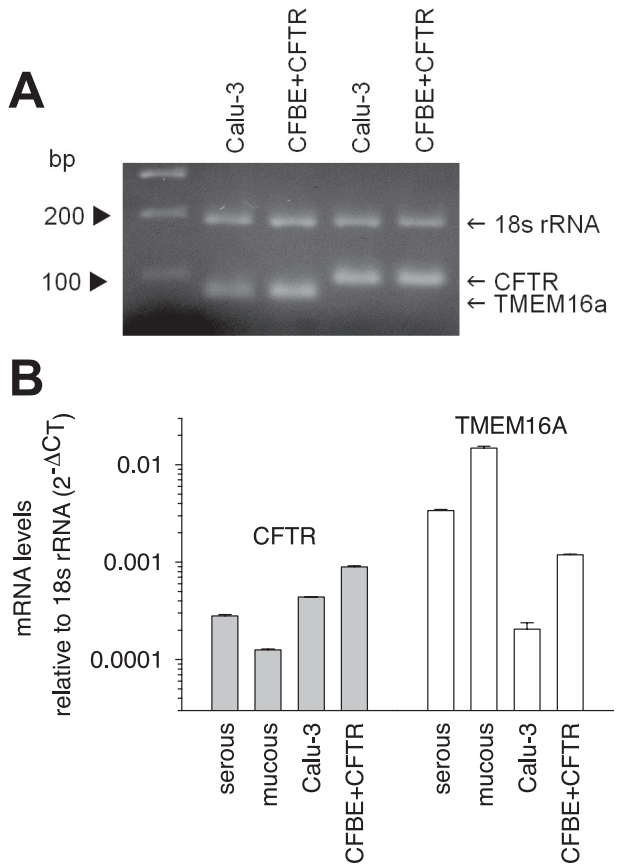

Fig. 5. mRNA levels of CFTR and transmembrane protein 16A (TMEM16A). *A*: specific amplification was verified using lysate from Calu-3 cells and CFBE41o$^-$ cells transfected with CFTR (CFBE+CFTR). *B*: mRNA levels relative to levels of 18S rRNA. Note log scale. C_T, cycle threshold.

bulk of the former. In several studies, we (28, 55) have shown an essentially linear dependence of I_{sc} on the protein or DNA content of surface epithelial cultures from human or cattle, and mucous cells have approximately double the protein (and DNA) content of serous (13).

In all Ussing chamber experiments, amiloride was given at the start of the experiment to abolish the contribution of active Na absorption to I_{sc}. It inhibited I_{sc} by 23 ± 7% in mucous cells and 12 ± 3% in serous. Based on previous studies in Calu-3 cells or in isolated glands, this was a surprising finding (26). However, both Calu-3 and submucosal glands have been shown to express ENaC subunits by PCR (26, 54). The functional significance of the amiloride-sensitive current for glands is currently unclear and requires further investigation. The remaining (amiloride-insensitive) I_{sc} is presumably due to anion secretion. We do not believe that HCO_3^- secretion makes a significant contribution to this because acetazolamide has negligible effects on I_{sc}, and base secretion measured by pH stat is only ~1 μA/cm^2 (B. Illek and H. Fischer, unpublished observations). Thus we assume amiloride-insensitive I_{sc} to represent predominantly active secretion of Cl. To estimate the contribution of CFTR to this, we used CFTRinh-172 (33). Added immediately after amiloride or at the end of the experiment, $CFTR_{inh}$-172 reduced I_{sc} by ~60% in both serous and mucous cells. By contrast, FFA, a blocker of CaCC, reduced baseline I_{sc} by ~20%. We conclude that CFTR-mediated Cl secretion accounts for the bulk of baseline Cl secretion in both serous and mucous cultures.

After amiloride, we added a series of pharmacological agents designed to elevate either intracellular cAMP or Ca. Qualitatively, serous and mucous cells behaved the same way: they gave transient I_{sc} responses to calcium-elevating agents (methacholine and ATP), which were blocked by incubation with BAPTA. In contrast, the cAMP-elevating agent, forskolin, was essentially without effect (Table 1). As with baseline I_{sc}, the mediator-induced responses of mucous cells were greater than those of serous. In serous cells, agonist responses were blocked partially by BAPTA, FFA, and $CFTR_{inh}$-172 (Fig. 1, *C* and *D*), consistent with roles for CaCC and CFTR in these responses. In mucous cells, $CFTR_{inh}$-172 inhibited the responses to methacholine by 64% and to ATP by 37%; FFA inhibited the ATP response by ~65% but had no effect on the response to methacholine. Thus methacholine-induced currents in mucous cells seem to be carried by CFTR with negligible contribution from CaCC, whereas ATP-induced currents are carried by both CaCC and CFTR with CaCC predominating. We speculate that these differences between the two agents reflect differences in the extent to which they elevate $[Ca]_i$ in key compartments within the tissue. Methacholine acts from the serosal but not the mucosal side (J. H. Widdicombe, unpublished observations). It may therefore activate basolateral K channels, hyperpolarize the apical membrane, and drive increased Cl current through constitutively open CFTR. However, changes in $[Ca]_i$ adjacent to the apical membrane may be insufficient to activate CaCC. By contrast, ATP stimulates Cl secretion from either side of the tissue (47) and may therefore raise $[Ca^{2+}]_i$ sufficiently to activate both apical membrane CaCC and basolateral K channels.

Forskolin had little or no effect on baseline Cl secretion. However, the inhibitory effects of $CFTR_{inh}$-172 suggested that the majority of this was mediated by CFTR. One possible explanation for the lack of effect of forskolin is that an increase in Cl conductance on opening of CFTR is accompanied by depolarization and an offsetting decrease in the driving force for Cl exit. In Ussing chamber experiments with chloride-free medium in the mucosal bath, any change in apical membrane voltage subsequent to opening of CFTR would have a comparatively small effect on the net electrochemical driving force for Cl exit. With mucosal Cl removed, forskolin was found to produce large increases in I_{sc}, which were abolished by GlyH-101 (Fig. 2), supporting the notion that driving forces across the apical membrane are small in symmetric Cl conditions, resulting in insignificant effects of forskolin. In fact, in the presence of a Cl gradient, responses to forskolin were now comparable in magnitude with those produced by ATP. Given that some of the Cl current induced by calcium-elevating agents is likely due to an increased driving force through constitutively open CFTR, this result supports the data from blockers suggesting that CFTR is quantitatively a more important contributor to baseline apical membrane Cl conductance than CaCC.

The suggestion that CFTR contributes more to apical membrane Cl conductance than CaCC is in good general agreement with results on intact glands. Thus comparing CF and non-CF tissues suggests that the relative importance of CFTR to CaCC in mediating pilocarpine-induced nasal gland fluid secretion is ~2:1 (42). Correcting for the fact that CF glands are hypertrophied (3) would increase this ratio. In fact, the effects of $CFTR_{inh}$-172 on non-CF glands suggest a ratio of ~8:1 (48). Other studies suggest the ratio of CFTR-mediated to CaCC-mediated gland secretion is ~2:1. Thus maximal secretory rates from human airway glands in response to carbachol were ~3 nl/min per gland and not significantly altered in CF (7). Of course, glands are ~3 times the normal volume in CF (3, 39), and correcting for this hypertrophy suggests that the CaCC-mediated gland secretion rate in non-CF tissues is ~1 nl/min. Two-thirds of the carbachol-stimulated secretion (i.e., 2 nl/min) would therefore be mediated by CFTR via a hyperpolarization of the apical membrane. In fact, this estimate of 2 nl/min is in good agreement with the maximal secretory rates in response to VIP. This agent acts through cAMP and induces maximal flow rates of ~1.5 nl/min per gland in non-CF glands but has no effect on CF glands (7).

A number of Cl channels have been described for the basolateral membrane of airway surface epithelium (14, 24), and we therefore used FFA to test whether basolateral CaCC could modulate Cl secretion by our tracheal gland cultures. This agent, added to the basolateral side, had no effect on Cl secretion or the response to methacholine but did have a small inhibitory action on the response to ATP. We are at a loss to explain this last result, given that inhibition of basolateral Cl channels should stimulate Cl secretion. Nevertheless, the results do argue against an important role for basolateral CaCC in regulation of Cl secretion across the cultures.

Perhaps the most surprising finding in our Ussing chamber studies was that baseline and mediator-induced levels of Cl secretion were greater in mucous than serous cell cultures. Thus it has generally been believed that serous cells secrete fluid that flushes out the mucus secreted by the more proximal mucous cells (36, 37), and even given the difference in bulk between the two cultured cell types, we expected serous cells to secrete more Cl than mucous. However, Wu et al. (53) have

recently observed fluid flows along the ducts of living human airway glands by differential interference contrast microscopy. On stimulation with forskolin, they found that estimated bulk flow accelerated in mucous tubules directly adjacent to the serous acini, consistent with fluid secretion by mucous cells. By contrast, flows were constant in the proximal unbranched duct, suggesting no secretion. Thus their results are consistent with our finding that not only serous, but also mucous cells show significant levels of Cl secretion in culture.

In our whole cell patch-clamp studies, the cAMP-activated conductance of serous cells was significantly greater, by ~3-fold, than in mucous or surface epithelial cells. This is consistent with the immunocytochemical finding that gland acini contain the highest levels of CFTR in the airways (10).

ATP had small effects on Cl currents in patch-clamp studies likely because the induced cellular Ca release was largely buffered by the EGTA contained in the pipette. Therefore, ionomycin was used to determine maximal CaCC currents. The ionomycin-stimulated currents were >10-fold larger than CFTR-mediated currents in serous, mucous, and surface cells. These CaCC currents were in part larger than can be accurately measured with whole cell patch-clamping, owing to the low membrane resistance and the associated poor voltage clamping of the membrane and the associated loss of Cl from the cell (34). This limitation in accurately measuring large whole cell currents may also explain the finding that mucous cells expressed 4.5 times more TMEM16A mRNA than serous cells; nevertheless, CaCC-mediated conductances were found to be not different in the 2 cell types (Table 2).

Despite these technical limitations, CaCC conductances in serous and mucous cells were significantly larger than in surface cells. This is consistent with the finding that cholinergic agents are the most potent secretagogues of airway glands (12). Furthermore, high levels for CaCC in serous and mucous cells support the evidence discussed above that both cell types contribute to mediator-induced gland secretion.

Interestingly, ionomycin had trivial effects on Cl secretion across polarized cell sheets. There is thus a major discrepancy in the results from the 2 systems. Calcium-activated Cl currents induced by ionomycin in whole cell patch-clamps were at least an order of magnitude greater than the cAMP-activated Cl currents, whereas in Ussing chambers, cAMP-dependent Cl secretion was somewhat greater than calcium-dependent even following addition of calcium-elevating agents. There are several possible explanations for these results. Perhaps, for some unknown reason, ionomycin is more potent at elevating $[Ca]_i$ in isolated cells than in polarized cell sheets. Perhaps in cell sheets CaCC is partitioned between apical and basolateral membranes in such a way that its opening results in approximately equal increases in Cl current across both membranes and no change in I_{sc}. However, the relative lack of effect of basolateral FFA on baseline or mediator-induced Cl secretion argues against this. We believe the most likely explanation is that there are quantitative differences between cell sheets and single cells in the trafficking of channels to the cell membrane. This was the conclusion reached by Anderson and Welsh (1). By selective permeabilization of the opposite membrane with nystatin, these authors were able to study the Cl conductance of either the basolateral or apical cell membrane of T84 cells. The apical membrane contained a large cAMP-activated Cl conductance but no calcium-activated Cl conductance. There was no calcium-activated Cl conductance in the basolateral membrane either. However, in whole cell patch-clamp studies, the calcium-activated Cl conductance was some 10-fold greater than the cAMP-activated. The same authors obtained similar results on human airway epithelial cell cultures; isolated cells had much higher calcium-activated Cl conductances than expected from the Ussing chamber studies. Furthermore, the calcium-activated Cl conductances in the apical membrane of permeabilized epithelia were voltage-independent but strongly rectified in whole cell patches. In short, the isolated cells used in patch-clamp studies expressed high levels of a calcium-activated Cl conductance not found in either the apical or basolateral membrane of polarized cell sheets. Anderson and Welsh (1) concluded that they were dealing with different Cl channels in cell sheets vs. single cells. In the studies reported here, FFA inhibited the calcium-activated currents in both systems, suggesting that we are dealing with quantitative differences in the expression of the same channel.

In conclusion, perhaps the most surprising finding of our studies was that the mucous cultures secreted more Cl than the serous. Some of this difference is ascribable to the relatively greater cell bulk of the mucous cultures. Nevertheless, the conclusion that mucous cells are an important contributor to overall gland secretion goes against generally accepted opinion. However, it is consistent with the somewhat limited information available from intact glands (53). Our second major conclusion is that in polarized cell sheets, CaCC and CFTR both contributed significantly to Cl secretion, with CFTR being somewhat more important than CaCC, a conclusion again consistent with results on intact glands. Finally, in contrast to the results on polarized cell sheets, in patch-clamp studies of isolated cells, we found that the maximal calcium-activated Cl conductance was ≥10 times greater than the maximal conductance activated by cAMP. We speculate that this reflects altered protein trafficking in isolated dispersed cells.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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