Exocytosis is not involved in activation of Cl− secretion via CFTR in Calu-3 airway epithelial cells

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Loffing, Johannes, Bryan D. Moyer, David McCoy, and Bruce A. Stanton. Exocytosis is not involved in activation of Cl− secretion via CFTR in Calu-3 airway epithelial cells. Am. J. Physiol. 275 (Cell Physiol. 44): C913–C920, 1998.—Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl− channel, which mediates transepithelial Cl− transport in a variety of epithelia, including airway, intestine, pancreas, and sweat duct. In some but not all epithelial cells, cAMP stimulates Cl− secretion in part by increasing the number of CFTR Cl− channels in the apical plasma membrane. Because the mechanism whereby cAMP stimulates CFTR Cl− secretion is cell-type specific, our goal was to determine whether cAMP elevates CFTR-mediated Cl− secretion across serous airway epithelial cells by stimulating the insertion of CFTR Cl− channels from an intracellular pool into the apical plasma membrane. To this end we studied Calu-3 cells, a human airway cell line with a serous cell phenotype. Serous cells in human airways, such as Calu-3 cells, express high levels of CFTR, secrete antibiotic-rich fluid, and play a critical role in airway function. Moreover, dysregulation of CFTR-mediated Cl− secretion in serous cells is thought to contribute to the pathophysiology of cystic fibrosis lung disease. We report that cAMP activation of CFTR-mediated Cl− secretion across human serous cells involves stimulation of CFTR channels present in the apical plasma membrane and does not involve the recruitment of CFTR from an intracellular pool to the apical plasma membrane.

Cystic fibrosis (CF), one of the most common lethal autosomal recessive genetic diseases in Caucasians, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a multifunctional, integral membrane protein (9, 32, 33, 41). CFTR is a cAMP-activated Cl− channel, which mediates transepithelial Cl− transport in a variety of epithelia, including airway, intestine, pancreas, and sweat duct (13). CF usually presents as exocrine pancreatic insufficiency, an increase in sweat Cl− concentration, male infertility, and airway disease. Airway disease leads to progressive lung dysfunction, which is the leading cause of morbidity and mortality in CF patients. Mutations in CFTR lead to impaired mucociliary clearance in the lung as a result of reduced cAMP-activated Cl− secretion by airway epithelial cells, hyperabsorption of sodium and water, and production of abnormal mucus and dehydration of the airways.

The lungs of CF patients become infected with Pseudomonas aeruginosa, setting in motion a cycle of inflammation, tissue damage, impaired lung function, and eventually death (5).

It is well accepted that stimulation of CFTR-mediated Cl− secretion by cAMP involves protein kinase A (PKA)-mediated phosphorylation of CFTR (13). In addition, in some but not all epithelial cells, cAMP also stimulates CFTR-mediated Cl− secretion by increasing the amount of CFTR in the plasma membrane. For example, in colon (T84 cells), kidney [Madin-Darby canine kidney (MDCK) II and A6 cells], and shark rectal gland, as well as bronchial epithelial cells, cAMP increases the amount of CFTR in the apical membrane (1, 11, 18, 27, 40). By contrast, in gall bladder and T84 cells1 cAMP does not increase the amount of CFTR in the plasma membrane (6, 29, 39).

Serous cells in human airway submucosal glands express high levels of CFTR, secrete antibiotic-rich fluid, and play a critical role in airway function (8). Dysregulation of CFTR-mediated Cl− secretion in serous cells is thought to contribute to the pathophysiology of CF lung disease (10, 35). However, it is not known whether cAMP regulates the amount of CFTR in the apical plasma membrane of serous cells. Because this issue has not been examined in serous cells and because the mechanism whereby cAMP increases CFTR-mediated Cl− secretion is cell-type specific, the goal of the present study was to determine whether cAMP elevates Cl− secretion across serous cells by increasing the amount of CFTR in the apical plasma membrane. To this end we studied Calu-3 cells, a human airway cell line with a serous cell phenotype (10, 12, 23, 35, 36). CI− secretion across Calu-3 cells is mediated by CFTR (10, 23, 36). We report that cAMP simulation of CFTR-mediated Cl− secretion across Calu-3 cells involves activation of CFTR channels present in the apical membrane and does not involve the recruitment and trafficking of CFTR Cl− channels from an intracellular compartment to the apical plasma membrane.

METHODS

Cell culture. Calu-3 cells were obtained at passage 17 (HTB-55, American Type Culture Collection, Rockville, MD), cultured in tissue culture flasks (Costar, Cambridge, MA) coated with Vitrogen plating medium (VPM) containing DMEM (JRH Biosciences, Lenexa, KS), human fibronectin

1 There is a discrepancy in the literature concerning the effect of cAMP on regulating the amount of CFTR in the apical plasma membrane in T84 cells (6, 29, 40). Although the reason is not clear, two points are relevant: 1) there are methodological differences among studies, and 2) immunolocalization of CFTR is technically challenging due in part to the low expression of CFTR in most epithelial cells (4, 42).
(10 µg/ml; Collaborative Biomedical Products, Bedford, MA), 1% Vitrogen 100 (Collagen, Palo Alto, CA), and BSA (10 µg/ml; Sigma Chemical, St. Louis, MO), and placed in an incubator maintained at 37°C and gassed with 5% CO2-95% air. Every 48 h, 90% of the medium (MEM, GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hyclidean, Logan, UT), 2 mM L-glutamine (GIBCO-BRL), 1 mM pyruvate, 50 U/ml penicillin, and 50 µg/ml streptomycin (Sigma Chemical) was replaced. At 90% confluence, cells were subcultured by incubation in Hanks’ balanced salt solution containing 0.05% trypsin and 0.53 mM EDTA and reseeded at a 1:7 dilution in VPM-coated Millicell PCF filters and grown in air-water interface culture (23, 35). The medium was completely changed every 24 h when cells were grown on Millicell filters. Cells between passages 25 and 34 were studied 10–15 days after seeding. At 24 h before Ussing chamber experiments, FBS was removed from the cell culture medium. In these growth conditions we observed a higher transepithelial resistance (Rt), and the cells appeared structurally more differentiated than was observed previously (35; see RESULTS). In addition, in these culture conditions we did not observe any oscillations in baseline short-circuit current (Isc), as reported previously (35).

Measurement of Isc. Isc was measured by placing monolayers grown on Millicell PCF filters into an Ussing-type chamber (Ijm’s Instrument, Iowa City, IA) and voltage clamping the transepithelial voltage (VT) across the monolayer to 0 mV with a voltage clamp (model VCC-600, Physiological Instruments, Poway, CA), as described previously (15, 16, 28). Bath solutions were maintained at 37°C and stirred by bubbling with 5% CO2-95% air (CO2/HCO3-containing solutions) or room air (CO2/HCO3-free solutions). Current output from the clamp was digitized by a TL-1 DMA Interface analog-to-digital converter (Axon Instruments, Foster City, CA). To measure RT, a 1.0-mV pulse (2-s duration) was imposed across the epithelium every 30 s, and the resulting current deflection was measured. RT was calculated by Ohm’s law: RT = ∆V/∆Isc, where ∆V is 1.0 mV. Data collection and analysis were done with Axotape 2.0 software (Axon Instruments).

In Ussing chamber studies, cells were bathed in an MEM (–FBS) solution gassed with 5% CO2, a CO2/HCO3-containing solution containing (in mM) 116 NaCl, 24 NaHCO3, 3 KCl, 2 MgCl2, 0.5 CaCl2, 3.6 HEPES (Na+ salt), and 4.4 H-HEPES (pH 7.4) gassed with 5% CO2-95% room air, or a CO2/HCO3-free solution containing (in mM) 116 NaCl, 24 sodium gluconate, 3 KCl, 2 MgCl2, 0.75 CaCl2 (to maintain Ca2+ activity constant due to chelation by gluconate), 3.6 HEPES (Na+ salt), and 4.4 H-HEPES (pH 7.4) gassed with room air and acetazolamide (100 µM) to inhibit the endogenous production of bicarbonate.

Immunofluorescence and confocal microscopy. Cells grown on Millicell PCF filters were immunostained for CFTR as previously described (6). Briefly, cells were fixed with 3% paraformaldehyde in PBS (Sigma Chemical) for 15 min, embedded in Tissue-Tek (Miles, Elkhart, IN), frozen in liquid propane that was cooled by liquid nitrogen, and stored at −80°C until further use. Sections (7–10 µm thick) were cut in a cryostat and placed on chrome alum gelatin-coated glass slides. Nonspecific binding sites were blocked with PBS containing 10% goat serum (PBS-GS, Dako, Carpinteria, CA), and sections were incubated with a 1:200 dilution of an anti-CFTR regulatory domain (IgG1) or anti-CFTR COOH-terminal (IgG2a) monoclonal antibodies (Genzyme, Cambridge, MA) in PBS with 0.5% BSA for 1–2 h at room temperature. Sections were washed with PBS-1% BSA and incubated with a 1:25 dilution of goat anti-mouse F(ab’2) fragment IgG-FITC (Dako) in PBS with 0.5% BSA for 1 h at room temperature. The immunolocalization pattern of CFTR with use of either antibody was identical. The regulatory domain antibody has been used by others to demonstrate that CFTR induces the recruitment of CFTR from a cytoplasmic pool to the apical plasma membrane (27, 40). Thus this antibody is capable of detecting changes in the intracellular localization of CFTR.

In some experiments, microtubules were stained with a monoclonal antibody against α-tubulin (Sigma Chemical) diluted 1:2,000 followed by a 1:25 dilution of a goat anti-mouse F(ab’2) fragment IgG-FITC (Dako) in PBS with 0.5% BSA. After they were repeatedly washed in PBS, cells were mounted in 1:1 PBS-glycerol containing 10 mg/ml n-propyl gallate (Sigma Chemical) as a fading retardant. In control sections the primary antibodies were omitted. To identify cell nuclei, nucleic acids were stained with propidium iodide (2.5 µg/ml). Sections were washed in PBS and mounted in DAKO-glycerol (Dako) containing 2.5% 1,4-diazabicyclo[2.2.2]octane as a fading retardant.

Fluorescent images were acquired using a microscope (Axioskop, Zeiss, Thornwood, NY) equipped with a laser scanning confocal unit (model MRC-1024, Bio-Rad, Hercules, CA), a 15-mW krypton-argon laser, and a ×63 PlanApochomat/1.4 numerical aperture (NA) or ×40 PlanNeofluor/1.3 NA oil immersion objective. FITC fluorescence was excited using the 488-nm laser line and collected using a standard FITC filter set (530 ± 30 nm). Propidium iodide fluorescence was excited using the 568-nm laser line and collected using a standard Texas red filter set (605 ± 32 nm). Acquired images were imported into Adobe Photoshop version 3.0 for image processing and printing.

Electron microscopy. Cells grown on Millicell PCF filters were fixed in 3.0% paraformaldehyde and 1.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4 adjusted to 330 mosM with sucrose) and stored in the fixative at 4°C until embedding into epoxy resin (Pluka, Buchs, Switzerland), as described previously (20). Briefly, filters were excised with a scalpel from the culture insert, postfixed for 15 min in 1% OsO4, dehydrated in a graded series of ethanol, washed in propylene oxide, and flat embedded into epoxy resin. Ultrathin sections were cut with an ultramicrotome (Reichert Jung, Vienna, Austria) and stained with lead citrate and uranyl acetate. Ultrathin sections were viewed and studied with a Phillips CM 100 electron microscope.

Apical membrane biotinylation. Biotinylation of apical cell membrane glycoproteins was performed as described in detail previously (19). All steps were performed at 4°C with gentle agitation. Cells grown on Millicell PCF filters were placed on...
Table 2. Effects of CO₂/ HCO₃⁻ on CPT-cAMP-stimulated $I_{sc}$

<table>
<thead>
<tr>
<th>Media</th>
<th>n</th>
<th>$V_N$</th>
<th>$R_N$</th>
<th>Basal</th>
<th>Peak after</th>
<th>Steady state after</th>
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<td>HCO₃⁻/CO₂⁻</td>
<td>13</td>
<td>2.5</td>
<td>300</td>
<td>9.4</td>
<td>41.9 ± 2.7</td>
<td>39.3 ± 2.2</td>
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<tr>
<td>HCO₃⁻/CO₂⁻ free + acetaza- zolamide</td>
<td>10</td>
<td>2.5</td>
<td>372</td>
<td>7.4</td>
<td>31.0 ± 3.1</td>
<td>21.0 ± 1.7</td>
</tr>
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</table>

Values are means ± SE; n, number of monolayers. All experiments were performed in the presence of amiloride (10 µM) in apical bath solution. $V_N$, transepithelial voltage; $R_N$, transepithelial resistance. HCO₃⁻/CO₂⁻, HEPES- and HCO₃⁻-buffered salt solution; HCO₃⁻/CO₂⁻ free, HEPES-buffered salt solution (see METHODS for composition). In preliminary experiments, we observed that acetazolamide added to HCO₃⁻/CO₂⁻-free solution had no effect on Cl⁻ secretion. *P < 0.05 vs. data in same column.

ice and washed three times with ice-cold PBS (with 1 mM CaCl₂ and 0.5 mM MgCl₂, PBS-Ca²⁺/Mg²⁺) to block vesicular trafficking. Sugar residues of apical plasma membrane glycoproteins were oxidized with sodium m-periodate (10 mM) in PBS-Ca²⁺/Mg²⁺ added to the apical cell surface for 30 min. After three washes with ice-cold PBS-Ca²⁺/Mg²⁺ and one wash with 0.1 M sodium acetate buffer (pH 5.5), oxidized apical glycoproteins were biotinylated with biotin-LC-hydrazide (2 mM; Pierce Chemical, Rockford, IL) in 0.1 M sodium acetate buffer (pH 5.5) added to the apical cell surface for 30 min. Subsequently, cells were washed three times with PBS-Ca²⁺/Mg²⁺ and fixed for detection of biotin by Texas red-conjugated streptavidin or processed for quantitation of apical cell surface CFTR. Control experiments were performed by omitting the biotinylation step.

After biotinylation, cells were lysed with 75 µl of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and protease inhibitors (Complete Protease Inhibitor Cocktail, Boehringer Mannheim, Indianapolis, IN), scraped from the filters, and transferred to an Eppendorf tube. Insoluble material was spun down by centrifugation at 14,000 g for 4 min. Supernatants were transferred to a new Eppendorf tube and brought to a total volume of 900 µl with lysis buffer, and biotinylated proteins were precipitated by adding 100 µl of a 50% suspension of streptavidin-agarose beads (Pierce). After overnight incubation at 4°C and continuous rotation, beads were pelleted by centrifugation for 30 s at 14,000 g and washed four times with lysis buffer. Biotinylated proteins were eluted from the beads by boiling for 5 min in 50 µl of Laemml sample buffer [0.2 M Tris-HCl (pH 8.9), 16% glycerol, 0.008% bromphenol blue, 5.6% SDS, and 80 mM dithiothreitol] including 3% EDTA to inhibit oxidation of dithiothreitol during sample boiling. Eluates were subjected to SDS-PAGE in precasted 4-15% polyacrylamide gels (BioRad) and transferred to polyvinylidene difluoride Immobilon membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C with 5% nonfat dry milk in Tris-saline (pH 8.0), 150 mM NaCl, 1% Tween 20, and one wash with 0.1 M sodium acetate buffer (pH 5.5) added to the apical cell surface for 30 min. Subsequently, cells were washed three times with PBS-Ca²⁺/Mg²⁺ and fixed for detection of biotin by Texas red-conjugated streptavidin or processed for quantitation of apical cell surface CFTR. Control experiments were performed by omitting the biotinylation step.

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peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL) diluted 1:5,000 in 5% nonfat dry milk and Tris-NaCl buffer. All antibody incubations were for 1 h at room temperature. After repeated rinsing, antibody-labeled proteins were visualized by the enhanced chemiluminescence detection method (Amersham) using Hyperfilm ECL (Amersham). Blots were digitally scanned, and the intensity of the bands was densitometrically analyzed with NIH Image version 1.57 software.

Statistical analysis. Differences between means were compared by unpaired Student's t-tests or ANOVA followed by Bonferroni's multiple comparisons test as appropriate. All analyses were performed with the InStat statistical software package (Graphpad, San Diego, CA). Values are means ± SE. P < 0.05 is considered significant.

RESULTS

8-(4-Chlorophenylthio)-cAMP stimulates electrogenic Cl− secretion by Calu-3 cells. In unstimulated monolayers of Calu-3 cells bathed in MEM (−FBS), Vt was −4.9 ± 0.6 mV, Rt was 341 ± 32 Ω·cm², and Isc was 19.6 ± 0.7 μA/cm² (n = 26). 8-(4-Chlorophenylthio)-cAMP (CPT-cAMP, 100 μM) rapidly increased Isc, which reached a peak value of 59.0 ± 3.0 μA/cm² (n = 26) in ~2 min and then decreased to a steady-state value of 51.0 ± 2.7 μA/cm² (n = 26). The Na+ channel blocker amiloride (10 μM) added to the apical bath solution had no effect on basal Vt, Rt, or Isc or on CPT-cAMP-stimulated Isc (n = 13). Thus the basal and CPT-cAMP-stimulated Isc was not referable to electrogenic Na+ transport. To determine whether the CPT-cAMP-stimulated Isc was mediated by CFTR Cl− channels, we examined the effects of the Cl− channel blockers diphenylamine-2-carboxylic acid (DPC), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), and DIDS added to the apical bath solution on the CPT-cAMP-stimulated Isc. DPC and NPPB reduced the CPT-cAMP-stimulated Isc to basal levels; however, DIDS had no effect on Isc (Table 1). Inhibition by DPC and NPPB but not by DIDS is a characteristic of CFTR Cl− channels (36).

In a previous study it was suggested that the DPC-sensitive, cAMP-activated Isc across Calu-3 cells was referable to HCO3−-dependent, CFTR-mediated Cl− secretion or Cl−-dependent HCO3− secretion (36). To determine whether CPT-cAMP stimulates Cl− secretion that is independent of HCO3−, we measured Isc across monolayers bathed in a CO2/HCO3−-free medium containing 100 μM acetazolamide to inhibit the endogenous production of HCO3−. Under these conditions, potential HCO3−-dependent Cl− secretion or Cl−-dependent HCO3− secretion is nominal. Removal of CO2/HCO3− reduced CPT-cAMP-stimulated Isc by 50% (Table 2). DPC and NPPB completely inhibited the CPT-cAMP-stimulated Isc. Thus ~50% of CPT-cAMP-stimulated Isc is mediated by DPC- and NPPB-sensitive Cl− secretion that is HCO3− independent.

Stimulation of Cl− secretion by CPT-cAMP is mediated by activation of CFTR Cl− channels in the apical plasma membrane. If CPT-cAMP stimulates Cl− secretion in part by recruiting CFTR Cl− channels from intracellular vesicles to the apical plasma membrane and/or by inhibiting the endocytotic retrieval of CFTR
from the apical membrane into cytoplasmic vesicles, activation of CFTR-mediated Cl\(^{-}\) secretion should be accompanied by or preceded by an increase in the amount of CFTR in the apical cell membrane. Figure 1 demonstrates that CPT-cAMP had no effect on the cellular localization of CFTR. CFTR was located almost exclusively in the apical cell membrane in control and CPT-cAMP-treated monolayers.

On the other hand, a reduction in CFTR Cl\(^{-}\) secretion may be mediated by relocating CFTR from the apical plasma membrane to intracellular vesicles and/or by inactivating CFTR channels in the membrane. If inhibition of Cl\(^{-}\) secretion is mediated by removal of CFTR from the apical membrane, then decreased Cl\(^{-}\) secretion should be accompanied by or preceded by a fall in the amount of CFTR in the apical cell membrane. It has been suggested that endogenous levels of cAMP are high in Calu-3 cells (35); thus it is likely that PKA is constitutively activated. Accordingly, to determine whether inhibition of Cl\(^{-}\) secretion is mediated by reducing the amount of CFTR in the apical membrane, we inhibited Cl\(^{-}\) secretion with H-89, a PKA inhibitor, and examined the effect of H-89 on \(I_{sc}\) and CFTR localization. H-89 (60 µM) decreased DPC-sensitive \(I_{sc}\) from 28.3 ± 2.8 to 13.3 ± 1.7 µA/cm\(^2\) \((n = 6, P < 0.01)\) after 60 min (Fig. 2). Although H-89 reduced Cl\(^{-}\) secretion by ~50%, inhibition of PKA had no effect on the cellular localization of CFTR (Fig. 1). In time-control experiments, vehicle had no effect on \(I_{sc}\) \((25.7 ± 2.8 \text{ vs. } 24.5 ± 3.9 \text{ µA/cm}^2; n = 5; \text{Fig. 2})\) or on the subcellular localization of CFTR. The observation that H-89 reduced Cl\(^{-}\) secretion is consistent with the view that basal cAMP levels are high in Calu-3 cells (35) and sufficient to activate PKA.

It is possible that modulation of Cl\(^{-}\) secretion by cAMP and H-89 may involve trafficking of CFTR Cl\(^{-}\) channels between the apical membrane and a submembranous cytoplasmic pool adjacent to the membrane (i.e., <0.2 µm). To test this hypothesis, we measured the amount of CFTR in the apical membrane by biotinylation and Western blot analysis. CPT-cAMP and H-89 had no effect on the amount of CFTR in the apical plasma membrane (Fig. 3). These observations suggest that CFTR is not stored in subapical cytoplasmic vesicles and inserted into the apical membrane after stimulation by CPT-cAMP. Electron micrographs are consistent with this view, inasmuch as there are few if any vesicles located below the apical membrane in Calu-3 cells (Fig. 4). The subapical, cytoplasmic region of Calu-3 cells is electron dense and lacks vesicles and cell organelles. By contrast, in cells where
agonists activate the rapid insertion of transport proteins (i.e., water channels, GLUT-4, or H^+ pumps) from an intracellular pool into the plasma membrane, the subapical cytoplasm contains numerous vesicles (2, 3).

Microtubules are not involved in acute upregulation of CFTR-mediated Cl^- secretion. The regulated trafficking of many proteins from an intracellular pool to the plasma membrane after an appropriate stimulus depends on intact microtubules (2, 3, 13, 40). Moreover, constitutive trafficking of proteins to the apical membrane is also microtubule dependent (17). To determine whether CPT-cAMP-activated Cl^- secretion is dependent on intact microtubules, we treated cells with nocodazole (33 µM for 3 h before addition of CPT-cAMP), a drug that blocks microtubule polymerization, or with taxol (10 µM for 3 h before addition of CPT-cAMP), a drug that stabilizes and perturbs microtubule function (3, 34). Nocodazole completely disrupted microtubules (Fig. 5) but had no effect on CPT-cAMP-stimulated I_sc (Table 3). Taxol caused microtubules to bundle and thicken, but it had no effect on CPT-cAMP-stimulated I_sc (Table 3, Fig. 5). Moreover, neither drug had an effect on the cellular localization of CFTR (Fig. 5). Scale bar, 5 µm.

Table 3. Effect of nocodazole or taxol on R_t and I_sc (Cl^- secretion) before and after CPT-cAMP in cells bathed in MEM-FBS

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>R_t, Ω·cm²</th>
<th>I_sc, µA/cm²</th>
<th>Peak after CPT-cAMP</th>
<th>Steady state after CPT-cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6</td>
<td>271 ± 17</td>
<td>23.4 ± 0.8</td>
<td>60.5 ± 1.8</td>
<td>53.3 ± 1.6</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>6</td>
<td>263 ± 8</td>
<td>32.0 ± 3.2*</td>
<td>61.9 ± 3.1</td>
<td>57.6 ± 2.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>3</td>
<td>327 ± 15</td>
<td>19.4 ± 0.7</td>
<td>48.9 ± 3.5</td>
<td>37.4 ± 1.0</td>
</tr>
<tr>
<td>Taxol</td>
<td>3</td>
<td>301 ± 14</td>
<td>20.3 ± 1.5</td>
<td>44.8 ± 2.0</td>
<td>36.6 ± 1.9</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of monolayers. We do not know why nocodazole increased basal I_sc. It is possible that disruption of microtubules for a prolonged period of time increased Ca^{2+}-dependent Cl^- secretion (23, 38). Additional studies, beyond the scope of the present study, are required to examine this possibility. FBS, fetal bovine serum. *P < 0.05 vs. in same column.
DISCUSSION

The major new finding of this report is that cAMP-mediated activation of CFTR-mediated Cl\textsuperscript{−} secretion across human airway serous epithelial cells (Calu-3) involves activation of CFTR channels present in the apical membrane and does not involve the recruitment of CFTR from an intracellular compartment to the apical plasma membrane. Neither cAMP nor the PKA inhibitor H-89 altered the amount of CFTR in the apical plasma membrane, yet these compounds dramatically influenced CFTR-mediated Cl\textsuperscript{−} secretion. The inability of the microtubule-disrupting agents nocodazole and taxol to alter the Cl\textsuperscript{−} secretory response to cAMP or the cellular localization of CFTR is also consistent with our conclusion that cAMP activation of CFTR-mediated Cl\textsuperscript{−} secretion across Calu-3 cells involves stimulation of CFTR channels present in the apical membrane.

Taken together with previous studies, our data are consistent with the conclusion that cAMP activation of CFTR-mediated Cl\textsuperscript{−} secretion is cell-type specific. In all cells examined, stimulation of CFTR by cAMP involves PKA-mediated phosphorylation of CFTR. However, in kidney (MDCK II and A6 cells) and shark rectal gland, as well as nasal and bronchial epithelial cells, cAMP also enhances Cl\textsuperscript{−} secretion by increasing the amount of CFTR in the apical plasma membrane (1, 11, 18, 27, 37, 40). By contrast, in gall bladder and colon (T84 cells), cAMP does not increase the amount of CFTR in the plasma membrane (6, 29, 39). It is well known that protein trafficking is cell-type specific, requiring numerous regulatory mechanisms and proteins that are expressed in some but not all cells (7, 26). For example, the human LDL receptor, when expressed in transgenic mice, is located in the apical membrane of renal tubules and in the basolateral membrane of colonocytes and enterocytes (3). The conclusion that the cellular mechanism whereby cAMP activates CFTR-mediated Cl\textsuperscript{−} secretion is cell-type specific has important implications for identifying therapeutic strategies to treat CF. Thus it is imperative that studies be conducted on epithelial cells affected by CF such as airway and pancreas, rather than on heterologous expression systems. Moreover, because the delivery of CFTR to the plasma membrane is dependent on the polarized state of epithelial cells, it is prudent to study polarized cells. For example, in unpolarized HT-29 intestinal cells, CFTR is localized in an intracellular compartment, whereas in polarized HT-29 cells, CFTR is sorted to the apical plasma membrane (24, 25).

Our studies with nocodazole and taxol also indicate that constitutive trafficking of CFTR in Calu-3 cells is low compared with other epithelial cells. For example, in T84 cells the half-life of CFTR in the plasma membrane is 2 min (30), and cAMP doubles the amount of CFTR in the apical membrane in 2 min (40). By contrast, in the present study on Calu-3 cells, impairment of microtubule function for 3 h by nocodazole or taxol had no effect on the amount of CFTR in the apical plasma membrane. Because disruption of microtubules blocks the intracellular flow of vesicles to the apical membrane of epithelial cells but has no effect on the recycling of early endosomes (14), our results with nocodazole and taxol suggest that CFTR has a very long half-life in the plasma membrane of Calu-3 cells (>3 h). This conclusion is supported by preliminary studies with brefeldin A, a fungal metabolite that inhibits the flow of vesicles from the endoplasmic reticulum to the Golgi apparatus and, thereby, blocks the delivery of newly synthesized proteins to the plasma membrane (34). We found that brefeldin A had no effect on cAMP-activated Cl\textsuperscript{−} secretion across Calu-3 cells.

Why does cAMP regulate Cl\textsuperscript{−} secretion in serous airway epithelial cells by activating channels that are present in the membrane? Calu-3 and serous cells have high basal rates of Cl\textsuperscript{−} and antibiotic secretion (23, 35). Thus relatively high and constant levels of CFTR in the membrane are required to mediate basal and cAMP-activated Cl\textsuperscript{−} secretion.

In conclusion, we report that stimulation of Cl\textsuperscript{−} secretion by human serous airway epithelial cells (Calu-3) involves activation of CFTR channels present in the apical plasma membrane and does not involve the recruitment of CFTR from an intracellular compartment to the apical plasma membrane. The observation that the cellular mechanism whereby cAMP activates CFTR-mediated Cl\textsuperscript{−} secretion is cell-type specific has important implications for studies directed at identifying therapeutic strategies for CF.

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