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Epinephrine stimulation of anion secretion in the Calu-3 serous cell model

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Banga A, Flaig S, Lewis S, Winfree S, Blazer-Yost BL. Epinephrine stimulation of anion secretion in the Calu-3 serous cell model. *Am J Physiol Lung Cell Mol Physiol* 306: L937–L946, 2014. First published April 4, 2014; doi:10.1152/ajplung.00190.2013.—Calu-3 is a well-differentiated human bronchial cell line with the characteristics of the serous cells of airway submucosal glands. The submucosal glands play a major role in mucociliary clearance because they secrete electrolytes that facilitate airway hydration. Given the significance of both long- and short-term β-adrenergic receptor agonists in the treatment of respiratory diseases, it is important to determine the role of these receptors and their ligands in normal physiological function. The present studies were designed to characterize the effect of epinephrine, the naturally occurring β-adrenergic receptor agonist, on electrolyte transport of the airway serous cells. Interestingly, epinephrine stimulated two anion secretory channels, the cystic fibrosis transmembrane conductance regulator and a Ca^{2+}-activated Cl^- channel, with the characteristics of transmembrane protein 16A, thereby potentially altering mucociliary clearance via multiple channels. Consistent with the dual channel activation, epinephrine treatment resulted in increases in both intracellular cAMP and Ca^{2+}. Furthermore, the present results extend previous reports indicating that the two anion channels are functionally linked.

cystic fibrosis transmembrane conductance regulator; transmembrane protein 16A; transepithelial ion transport; airway epithelia; mucociliary clearance

β-ADRENERGIC RECEPTOR AGONISTS (β-agonists) are widely used in the treatment of respiratory ailments such as asthma and chronic obstructive pulmonary disease (COPD). β-Agonists cause vasodilation via an effect on smooth muscle, and the target tissue for clinical intervention is predominately vascular. However, with inhaled medication, it is inevitable that other cell types will be exposed to these drugs, potentially causing a number of other effects.

In the lung, β-adrenergic stimulation enhances fluid absorption, thereby aiding in resolution of pulmonary edema after birth or lung injury (3, 9, 30). This effect is mediated, at least in part, by stimulation of the epithelial Na^+ channel (ENaC), resulting in net ion and fluid absorption (18). In the submucosal glands of the airways, however, the response to adrenergic stimuli is quite different. In the glands, the increased cAMP resulting from β-adrenergic receptor stimulation results in a stimulation of ion and fluid secretion (12, 19), thereby facilitating mucociliary clearance. One of the predominate channels involved is the Cl^-/HCO_3^- anion channel cystic fibrosis transmembrane conductance regulator (CFTR).

The long-acting β-agonists that are widely used for clinical treatment may have chronic actions on CFTR expression and/or translocation to the apical membrane (36). However, the natural, short-acting effectors such as epinephrine are not widely studied, and there is a paucity of information regarding the action of the natural ligand of the β-adrenergic receptor in airway epithelial cells. Because epinephrine is unique as a homeostatic mediator, functioning as both a neurotransmitter and a hormone, such studies will facilitate an understanding of the role of epinephrine in both normal and pathological regulation.

Calu-3 is a well-differentiated and well-characterized human bronchial cell line derived from a lung adenocarcinoma (8, 35). This cell line has the characteristics of the human airway serous cells of submucosal glands that are known to secrete electrolytes and water involved in hydrating airway surfaces. The serous glands are important for continuous mucociliary clearance from the airways, and the lack of CFTR in these glands in patients with CF causes the dehydrated secretions that are a hallmark of this disease (10, 31, 37, 40).

The Calu-3 cell line forms polarized monolayers with tight junctions and exhibits high transepithelial electrical resistances and high basal ion transport (35). This cell line has been shown to exhibit rapid onset anion secretion when exposed to mediators like isoproterenol, forskolin, bradykinin, methacholine, trypsin, and histamine, indicating that both cAMP and Ca^{2+}-stimulated ion transport processes are present in these cells (35). The Calu-3 cells contain CFTR, which exhibits Cl^-/HCO_3^- secretion when stimulated via the adenylyl cyclase/adenosine 3′,5′-cyclic monophosphate (cAMP)/protein kinase A (PKA) pathway (35). Calu-3 cells also express mRNA and activity consistent with transmembrane protein 16A (TMEM16A) (ANO1), a recently described Ca^{2+}-activated Cl^- channel (CaCC) (11). Although not as thoroughly studied as CFTR, TMEM16A has been described as the predominant CaCC in airways (26). Furthermore, in airway epithelial cells, it has been suggested that there are functional and molecular interactions between CFTR and TMEM16A (25).

Epinephrine occupies β-adrenergic receptors, increases cAMP levels, and has been shown to stimulate anion efflux in Calu-3 cells (2, 19). Calu-3 cells contain β_2-adrenoceptor (β_2-AR) mRNA and protein. Stimulation with the adrenergic effector isoproterenol increased cAMP production, an effect that was blocked by a β_2-antagonist but not a β_1-antagonist (1). Subsequent studies showed a physical interaction between CFTR and β_2-AR, which were linked via PDZ domains with ezrin/radixin/moesin-binding phosphoprotein 50 (24).

We have now further characterized the response of the Calu-3 cells to epinephrine. The results indicate the activation of both CFTR and TMEM16A in response to the naturally occurring hormone. The data indicate that effects on serous cells should be considered when treating other respiratory target tissues with either short- or long-term β_2-AR agonists.

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MATERIALS AND METHODS

Materials. Cell culture flasks and Transwell cell culture plates (24-mm inserts, polycarbonate, 0.4-μm pore size) were obtained from Costar-Corning (Acton, MA). Trypsin-EDTA solution and Hanks Balanced salt solution (HBSS) were obtained from Mediatech (Herndon, VA). Dulbecco's modified Eagle's medium/Hams F-12 (DMEM/F12) tissue culture media, Glutamax, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was obtained from Gemini Bioproducts (West Sacramento, CA). Ionomycin, BAPTA-AM, and the Direct Cyclic AMP Enzyme Immunoassay Kit were from Enzo Life Sciences (Farmingdale, NY). Epinephrine was purchased from BioData (Horsham, PA). Amiloride and tannic acid were obtained from Sigma Chemical (St. Louis, MO). Forskolin and 5-nitro-2-(3-phenyl-propylamino) benzoate (NPPB) came from Biomol International (Plymouth Meeting, PA), and CFTR inh172 was purchased from Calbiochem-Merck (Darmstadt, Germany). $T16A_{inh}$-AO1 was provided by Prof. Alan Verkman. Anti-TMEM16A antibodies were purchased from Novus Biologicals (Littleton, CO) and Aviva Systems Biology (#ARP42506; San Diego, CA). AlexaFluor 488 goat anti-rabbit secondary antibody, fura 2-AM, wheat germ agglutinin (WGA) 555, and Cell Mask Orange plasma membrane stain were obtained from Life Technologies Invitrogen (Grand Island, NY).

Cell culture. The Calu-3 cell line, passage 19 (no. HTB-55), was purchased from ATCC (Manassas, VA). Cells were cultured in 75-cm^2 tissue culture flasks and grown in a humidified atmosphere of 5% CO_2 at 37°C. Cell culture medium was comprised of DMEM/F-12 with 2.40 g/l $NaHCO_3$ and 1 mM Glutamax. The medium was supplemented with 15% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.5 mM nonessential amino acids, and 0.5 mM sodium pyruvate. The medium was replaced three times per week, and cells were passaged weekly at a split ratio of 1:4 using 0.5% trypsin-EDTA solution. For electrophysiology, the cells were seeded directly onto Transwell inserts composed of polycarbonate permeable filters with media on the apical and basolateral sides. Two days after inoculation of the Calu-3 cells, the media were removed and replaced only on the basolateral side (air interface culture; AIC). Cells grown to confluent monolayers were used on the 14th-16th day after being seeded onto the Transwells, the time at which the cells show a high-resistance phenotype. As previously reported, there is a batch-to-batch variability in the cells (7, 16, 38); therefore, in all experimental protocols, control and treated cultures from the same seeding were analyzed in parallel. The average transepithelial resistance for each batch of cells varied between 500 and 700 Ω/cm^2. Cultures with resistances below 500 Ω/cm^2 were not used for experiments.

Electrophysiology. Electrophysiological techniques were used to monitor changes in ion flux across the cellular monolayers in response to epinephrine stimulation. The filters were cut from the plastic inserts and mounted in an Ussing chamber that separates the apical (mucosal) surface from basolateral (serosal) surface. Ussing chambers were connected to a DVC-1000 voltage/current clamp (World Precision Instruments, Sarasota, FL) with voltage and current electrodes on either side of the membrane. The cells were bathed in serum-free medium maintained at 37°C via water-jacketed buffer chambers on either side of the filter. Media were circulated and kept at constant pH using a 5% CO_2-95% O_2 gas lift. The spontaneous transepithelial potential difference was clamped to zero, and the resultant short-circuit current (SCC) was monitored continuously. Under these conditions, SCC is a measure of net transepithelial ion flux. By convention, an increase in SCC denotes either anion secretion or cation absorption. After the basal current stabilized (30–60 min after mounting; time = 0), epinephrine or forskolin were added to the serosal bathing medium. Amiloride, tannic acid, NPPB, $T16A_{inh}$-AO1, and CFTR inh172 were added apically. $BaCl_2$ was added to either side of the confluent monolayer as indicated in the pertinent figure. Preincubation with ionomycin or BAPTA was accomplished by the apical addition of the intracellular Ca^{2+} effectors.

cAMP assay. Calu-3 cells were grown to confluence on Transwell supports and stimulated with epinephrine (10^{-6} M) for either 20 or 60 s. Each culture was washed twice with 37°C HBSS and lysed by a 10-min incubation with 1% Triton X-100 in 0.1 M HCl at 37°C. Cells were scraped from the Transwells, and the lysates were centrifuged for 1 min at 14,243 *g* to remove cellular debris. cAMP concentrations were determined using the Direct Cyclic AMP Enzyme Immunoassay Kit according to the manufacturer instructions and expressed as picomoles per square centimeter of confluent cells.

Visualization of intracellular Ca^{2+}. Calu-3 cells were grown to confluence on Transwell supports (14–16 days), stained with 5 μM fura 2-AM for 30 min in HBSS or HBSS++ (+ calcium and magnesium) for epinephrine or ionomycin treatment, respectively, at 37°C with 2.5 mM probenicid to enhance fura 2 retention. Monolayers were then washed with HBSS, stained for 5 min with CellMask Orange 1 μM, excised, and placed apical surface down onto a 35-mm coverglass bottom dish followed by addition of HBSS or HBSS++ to maintain hydration of the membrane. Dishes (35 mm) were mounted in a stage-top incubator to maintain temperature at 37°C (OKOLab, Pozzuoli, Italy). Monolayers and fields were selected with the CellMask Orange staining, and the fura 2 was imaged every 2 s with both 340-nm and 380-nm excitation for 30 s before stimulation with either epinephrine or ionomycin and imaged for 5 min after stimulation. Imaging was performed on a custom epifluorescent microscope with a heated S Fluor 40 × 1.3 NA objective lens built around a TiE microscope stand fitted with Perfect Focus (OKOLabs/Nikon Instruments) motorized stage (Prior Scientific, Rockland, MA), a Lambda LS Xenon light source (Sutter Instruments, Novato, CA), an ORCA ER interline CCD camera (Hamamatsu Phototonics, Hamamatsu, Japan) and controlled by NIS Elements AR v 4.10 (Nikon Instruments, Tokyo, Japan). Filters used included the ET fura 2 and Sedat Quad (Chroma Technology, Bellows Falls, VT).

All postacquisition analysis was performed in Fiji v1.48p (32). The change in Ca^{++} was followed by calculating the ratio of fura 2 emission at 510 nm with 340 nm and 380 nm excitation, R340/380.

Immunolocalization of TMEM116A. Calu-3 cells were grown on Transwell supports for 14–16 days, washed with PBS, and fixed in 4% paraformaldehyde for 20 min at room temperature. Subsequently, the fixed, confluent monolayers were incubated with 75 mM ammonium chloride/20 mM glycine in PBS to quench free aldehyde groups. The cells were incubated in WGA-fluorophore conjugate before permeabilization to stain the plasma membrane but not intracellular organelles. Cells were blocked and permeabilized with SS-PBS (10% normal goat serum, 0.1% saponin in PBS). The cells were stained with primary antibody for 2 h at room temperature followed by AlexaFluor 488 goat anti-rabbit secondary antibody.

Samples were imaged by confocal fluorescence microscopy on an upright TCS SP8 with resonant scanner with a 25 × 0.95 NA water immersion objective lens (Leica, Jena, Germany). Imaging was performed sequentially with excitation provided by two solid-state lasers at 488 nm and 552 nm, and emission was collected on HyD detectors (Leica) with spectral filtration from 489–552 nm and 561–741 nm, respectively. Confocal slices were taken at 1-μm intervals.

Statistics. Statistical comparisons were done using Student's *t*-test. The cut-off parameters are listed in the individual figure legends.

RESULTS

Calu-3 cells were grown as an AIC to simulate the in vivo environment. Under AIC conditions, Calu-3 cells form well-developed tight junctions, polarize, develop a high-resistance phenotype, and express CFTR (35). The transcription profile of AIC-grown Calu-3 cells was found to more closely resemble that of in vivo airway epithelia than cells grown in submerged

cultures (27). All of the present studies were performed in cells grown under AIC conditions.

Calu-3 cells have been shown to respond to a wide variety of hormones and effectors, but, despite the presence of β_2-AR in this cell line (1), few studies have been performed to characterize the cellular response to the naturally occurring hormone, epinephrine. Calu-3 monolayers were grown on permeable supports, mounted in Ussing chambers, and allowed to reach a stable baseline. Addition of epinephrine to the serosal side of the monolayer produced a multifaceted response that was initiated by a rapid and transient increase in ion flux followed by progressively dampening oscillations in ion fluxes over time (Fig. 1). The cyclic nature of the response has been described previously in response to effectors that increase intracellular cAMP (21, 35, 38). However, it is important to note that this type of response is not uniformly described in publications examining compounds that stimulate ion transport in Calu-3 cells. In our experiments, the nadir of the ion flux oscillations is higher than the basal (prestimulated) level, indicating a sustained increase in ion transport.

To confirm that epinephrine signals via the adenylyl cyclase pathway, we measured the cAMP levels 20 and 60 s after the addition of the hormone. Epinephrine stimulated a rapid nine- to tenfold increase in cAMP over the control levels (Fig. 2).

Both Cl^- secretion via CFTR and Na^+ absorption via the ENaC are positively stimulated by increases in cAMP. To determine whether basal or epinephrine-stimulated current was attributable to transepithelial Na^+ flux via ENaC, the cells were pretreated with amiloride, a specific ENaC inhibitor. Neither the basal nor the stimulated current was inhibited by amiloride. The residual ion transport remaining 20 min after epinephrine stimulation was inhibited by NPPB, a nonspecific Cl^- channel blocker (Fig. 3). Basal current (before epinephrine stimulation stimulation) was also substantially inhibited by NPPB (data not shown). These observations indicate that the basal and epinephrine-stimulated ion transport are predominately attributable to anion secretion.

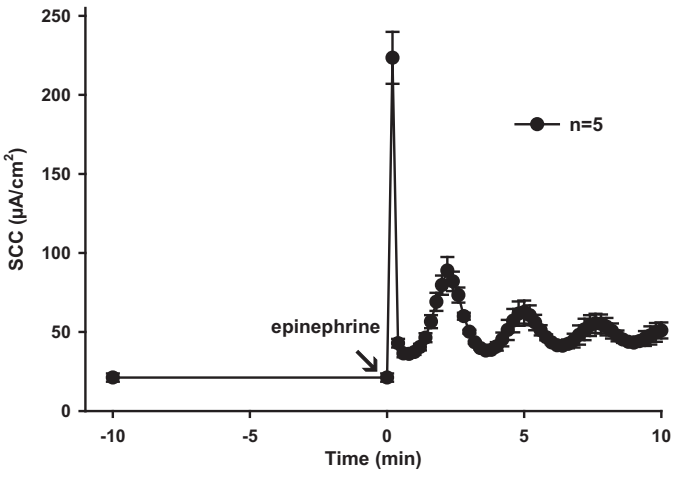

Fig. 1. Response of Calu-3 cells to epinephrine. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable basal short-circuit current (SCC). SCC is a measure of net transepithelial ion transport. After stabilization, epinephrine (10^{-6} M) was added to the serosal bathing media (time, $t = 0$). The symbols denote the means of 5 experiments ± SE.

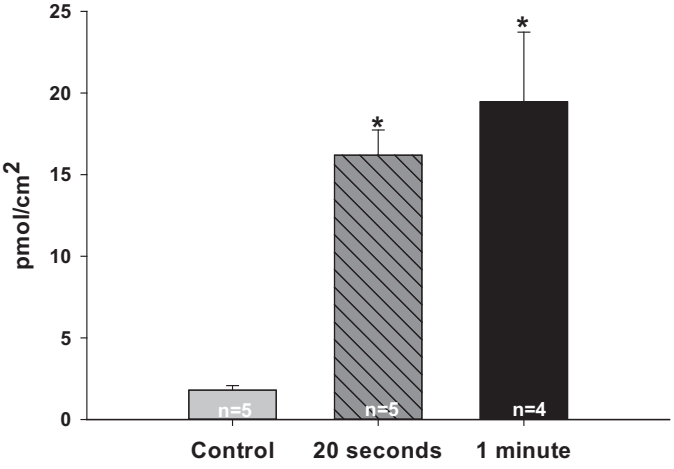

Fig. 2. cAMP production in response to epinephrine stimulation. cAMP levels were measured in cellular lysates 20 and 60 s after stimulation of the intact monolayer with epinephrine (10^{-6} M). The numbers at the base of the columns represent the number of independent experiments performed. The cAMP is expressed as picomoles per square centimeter of confluent cells that had been cultured on permeable filter supports (Transwells). Values on the bars are means ± SE. *Statistically higher than the control values, $P < 0.02$, 2-tailed Student's t-test.

An alternative method to activate intracellular cAMP is to constitutively stimulate the first enzyme of the pathway, adenylyl cyclase, with forskolin. Forskolin addition to Calu-3 cells resulted in a stimulation that mimicked the oscillations in ion flux that are typical of epinephrine stimulation but did not produce the initial transient ion flux of the hormonal stimulation. This is not due to variability in the cultures because epinephrine elicited a robust initial peak in a parallel culture (Fig. 4). Addition of epinephrine to forskolin-pretreated cells enhanced the oscillating current that was diminishing after the forskolin effect but did so in the absence of the initial, transient

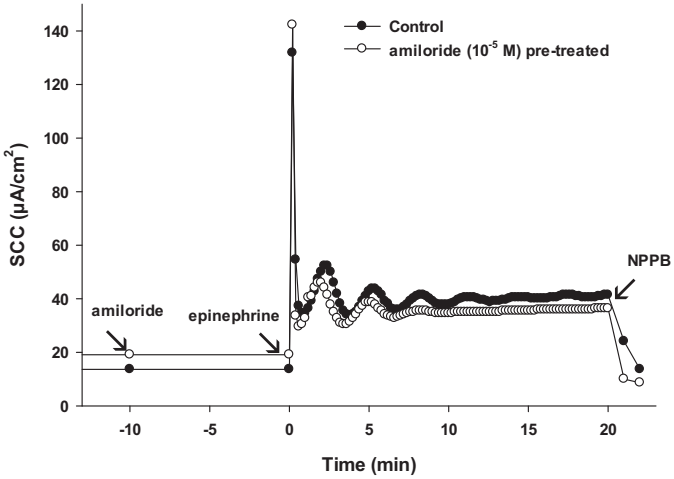

Fig. 3. Effect of amiloride pretreatment on the epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable basal SCC. ●, Control; ○, cells pretreated with amiloride (10^{-5} M) for 10 min. At $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of both cultures. At $t = 20$ min, 5-nitro-2-(3-phenyl-propylamino) benzoate (NPPB) (100 μM) was added to inhibit the Cl^- secretion. The plots show single, representative experiments performed on cells grown in parallel.

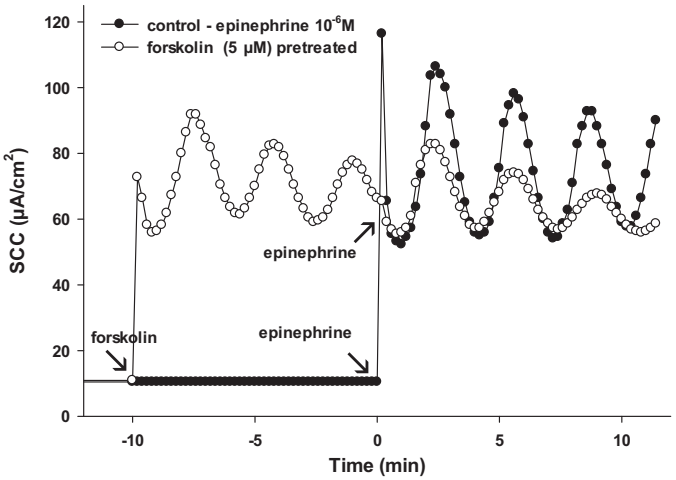

Fig. 4. Effect of forskolin on the Calu-3 cell line and on a subsequent response to epinephrine. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable basal SCC. ●, Control; ○, cells treated with forskolin (5 μM) at $t = -10$ min. At $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of both cultures. The plots show single, representative experiments performed on cells grown in parallel.

transport (Fig. 4). Thus it appears that the responses to forskolin and epinephrine differ. These data suggest that epinephrine stimulation may activate more than one Cl^- transport process.

On the basis of the epinephrine-stimulated increase in cAMP, the most likely Cl^- secretory channel is CFTR. CFTR inh172 is a specific inhibitor of CFTR (20). Exposure of the Calu-3 cells to CFTR inh172 significantly decreased the basal transport rate (Fig. 5*B*) and subsequently partially inhibited the initial transient peak. In addition, the oscillatory ion-transport events were completely abolished although a sustained level of transport over control basal level was maintained in the presence of the CFTR inhibitor (Fig. 5*A*).

There is a substantial literature showing that CaCCs exist in airway epithelial in general and in Calu-3 cells in particular (11, 21, 22, 25–28, 38). A prime candidate for the CaCC is the recently elucidated TMEM16A or ANO1 (5, 33, 41). Tannic acid is a widely used inhibitor of TMEM16A that does not directly modulate activity of the CFTR chloride channel (22). Like CFTR inh172, tannic acid caused a transient decrease in the basal transport rate (Fig. 6*B*). Tannic acid partially inhibited the initial transient peak but had no effect on the oscillatory fluxes or the sustained increase in transport (Fig. 6*A*).

Coaddition of both the CFTR inhibitor and tannic acid caused a slight addition stimulation that is often detected immediately after the addition of CFTR inh172. Thereafter the basal transport was diminished more than the inhibition caused by either agent alone (Fig. 7*B*). Epinephrine stimulation caused a slight increase immediately after addition, but this rapidly dissipated, resulting in inhibition of all components of the response, namely the initial transient anion secretion, the oscillatory ion flux, and the sustained increased level of transport above baseline. Compared with either inhibitor alone, the inhibitory effects of the two agents appear to be synergistic.

Previous studies have suggested that tannins may inhibit both cAMP- and Ca^{2+}-stimulated pathways in bovine airway epithelia (6); therefore, we performed electrophysiological studies using a more specific inhibitor of TMEM16A, $T16A_{inh}$-A01 (21). The data, shown in Fig. 8, indicate that preincubation with this inhibitor decreased basal as well as all epinephrine-stimulated transport.

To further substantiate that the CaCC contributing to the epinephrine-stimulated Cl^- secretion was TMEM16A, confocal immunohistochemistry was used to determine the presence and localization of the channel. Figure 9 illustrates the results using antibodies produced by both Novus Biologicals and Aviva Systems Biology. Both of these antibodies were raised against synthetic peptides from the human TMEM16A sequence. The Novus antibody was raised against a peptide from the NH_2-terminal domain, whereas the Aviva antibody was directed against the middle portion of the protein.

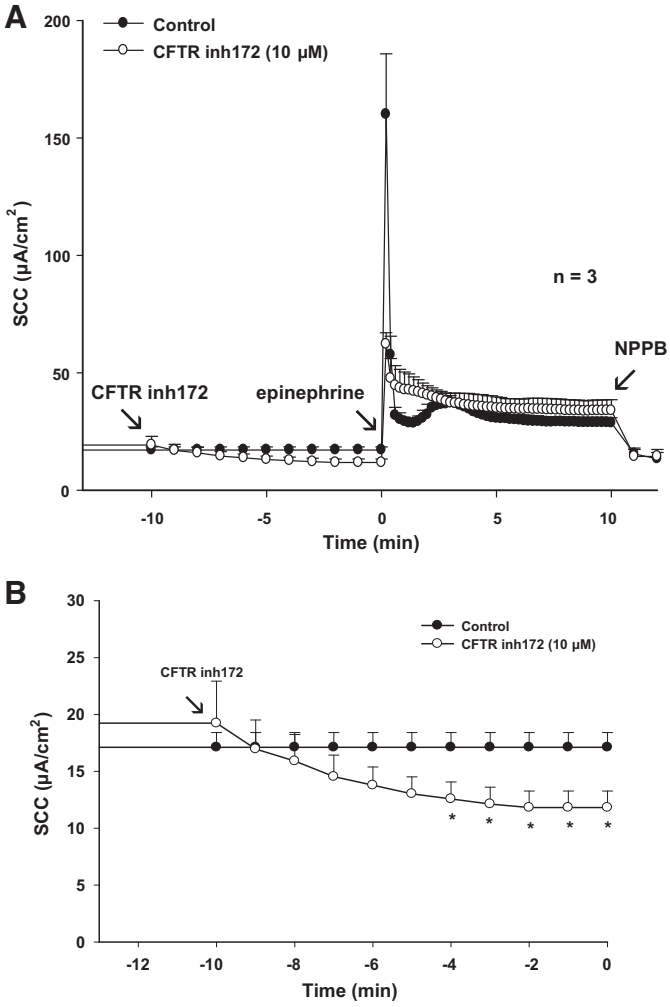

Fig. 5. Effect of cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor 172 (inh172) pretreatment on the epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable basal SCC. The graphs depict the continuous measurement of SCC, a measure of net ion transport of the cells. ●, Control; ○, cells pretreated with CFTR inh172 (10 μM) for 10 min. *A*: at $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of all experiments. At $t = 20$ min, NPPB (100 μM) was added to inhibit Cl^- secretion. The symbols on each plot denote the means of 3 experiments ± SE. *B*: expanded view of the effect of adding the CFTR inhibitor, CFTR inh172. *Statistically lower than the control baseline, $P < 0.05$, 1-tailed Student's *t*-test.

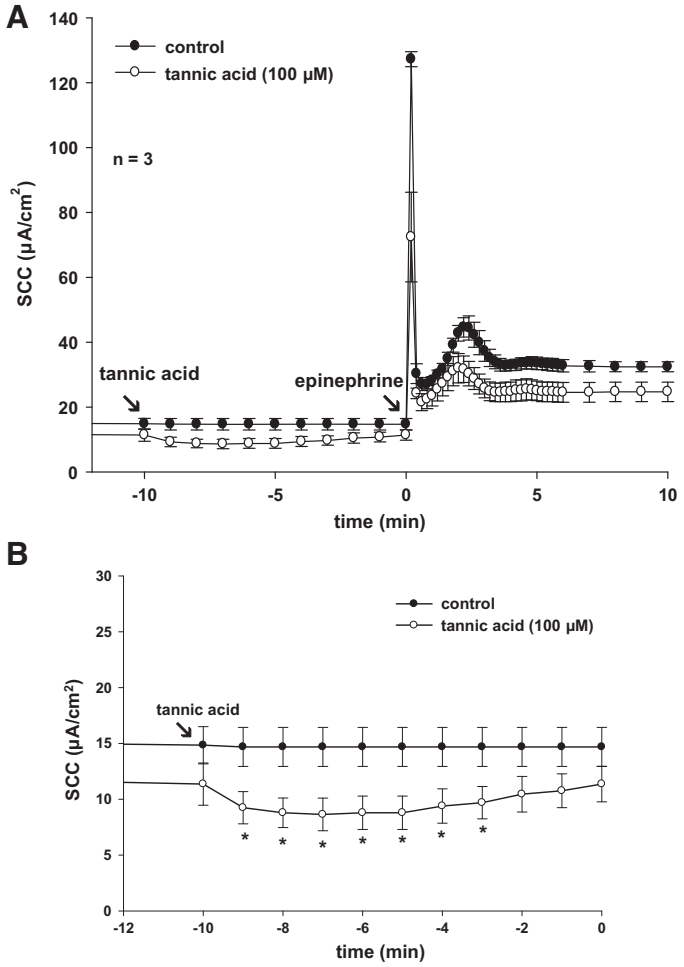

Fig. 6. Effect of tannic acid pretreatment on the epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable basal SCC. The graphs depict the continuous measurement of SCC, a measure of net ion transport of the cells. ●, Control; ○, cells were pretreated with tannic acid (100 μM) for 10 min. *A*: at $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of all experiments. The symbols on each plot denote the means of 3 experiments ± SE. *B*: expanded view of the effect of adding the transmembrane protein 16A (TMEM16A) inhibitor, tannic acid. *Statistically lower than the control baseline, $P < 0.05$, 1-tailed Student's *t*-test.

Both antibodies recognize epitopes, which were predominately on the apical membrane in the permeabilized cells. The Novus antibody, directed against the NH_2-terminal domain, showed a more intense staining pattern. The Aviva antibody, although not demonstrating as high an affinity, showed specific labeling when compared with the control with no primary (Fig. 9, *right*).

To determine whether the epinephrine stimulation produced an increase in intracellular Ca^{2+}, Calu-3 cells were preincubated with fura 2. As a positive control, some cells were stimulated with ionomycin, a Ca^{2+}-selective ionophore that raises intracellular Ca^{2+} in the absence of other effectors. As shown in Fig. 10, *middle*, the ionomycin caused a maximal increase in intracellular Ca^{2+}. The more physiological situation is shown in Fig. 10, *top*, where epinephrine stimulation caused a more modest increase in intracellular Ca^{2+}. The average R340/380 excitation ratio of the whole field shown in Fig. 10, *top, left*, is a relative measure of the intracellular Ca^{2+}. The increased concentration of intracellular Ca^{2+} is maintained for the entire 5 min of imaging (data not shown). Additional experiments were performed to substantiate the role of intracellular Ca^{2+}.

Addition of ionomycin to the Calu-3 cells increased the basal transport rate substantially (Fig. 11). Subsequently, stimulation with epinephrine resulted in a slightly attenuated initial transport rate, an enhanced oscillatory ion flux, and a higher sustained basal ion flux. Other, more subtle, changes are also created by the increased intracellular Ca^{2+} concentration. The initial peak, is more prolonged but the oscillations in ion flux show an increased periodicity (Fig. 11).

1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) is a cell-permeable Ca^{+2} chelator. The addition of BAPTA-AM caused a slight decrease in basal transport (not statistically significant) and almost

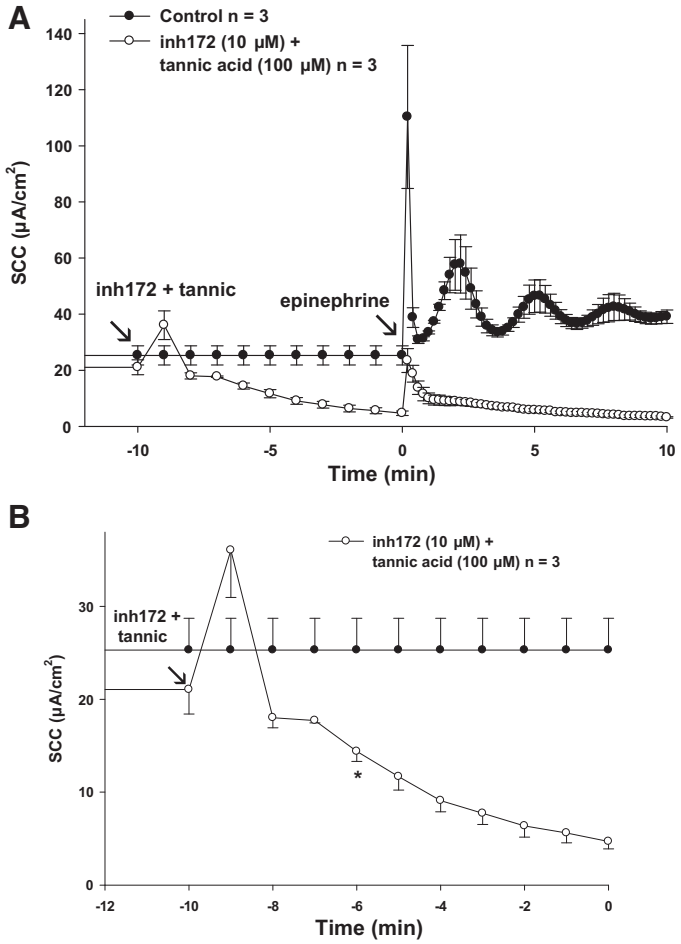

Fig. 7. Effect of CFTR inh172 and tannic acid pretreatment on the epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable basal SCC. The graphs depict the continuous measurement of SCC, a measure of net ion transport of the cells. ●, Control; ○, cells were pretreated with CFTR inh172 (10 μM) and tannic acid (100 μM) for 10 min. *A*: at $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of all experiments. The symbols on each plot denote the means of 3 experiments ± SE. *B*: expanded view of the effect of adding the TMEM16A inhibitors. *All values at and after this point are statistically lower than the control baseline, $P < 0.05$, 2-tailed Student's *t*-test.

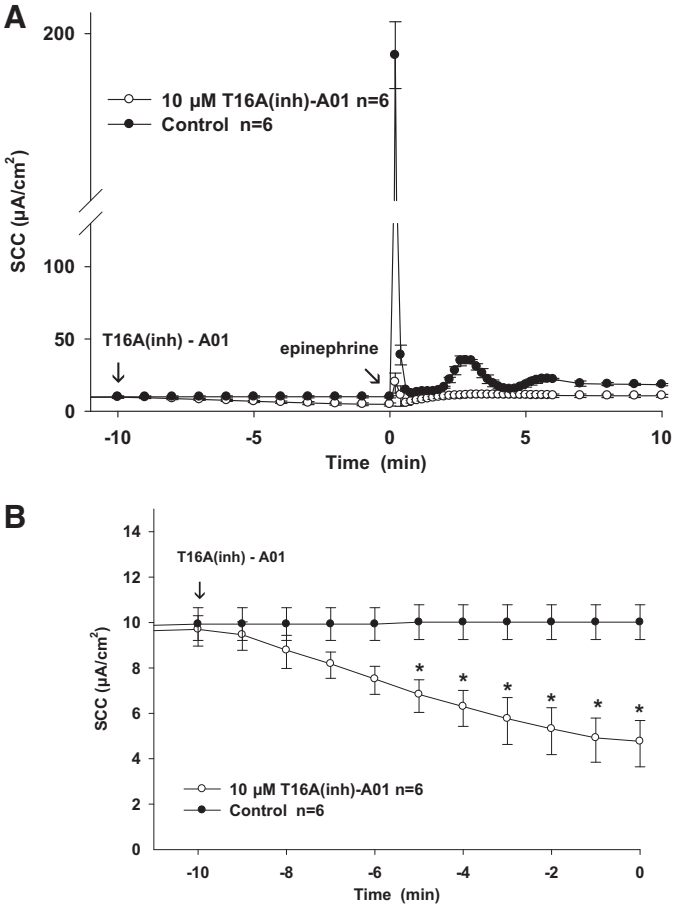

Fig. 8. Effect of $T16A_{inh}$-A01 on epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14–16 days, excised, mounted in an Ussing chamber, and allowed to develop a stable SCC. The graphs depict the continuous measurement of SCC, a measure of net ion transport of the cells. ●, Control; ○, cells were pretreated with $T16A_{inh}$-A01 (10 μM) for 10 min. *A*: at $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of all experiments. The symbols on each plot denote the means of 6 experiments ± SE. *B*: expanded view of the effect of adding the TMEM16A inhibitor. *Statistically lower than the control baseline, $P < 0.05$, 2-tailed Student's *t*-test.

completely blocked epinephrine-stimulated transport. The only remaining transport is a rather flat level of sustained ion flux compared with the basal rate (Fig. 12).

Many previous studies have indicated the important contribution of both basolateral and apical potassium channels for establishing and maintaining the driving force for anion secretion. In keeping with those studies (e.g., Refs. 21 and 38), $BaCl_2$, a nonspecific inhibitor of K^+ channels, had a minor effect on baseline and a significant inhibitory effect on both the initial secretory current and the oscillatory currents but only when added to the basolateral face of the cells (Fig. 13).

DISCUSSION

Homeostatic control of fluid secretion and absorption in the respiratory system is crucial and complex. In general terms, ENaC-mediated Na^+ absorption is accompanied by osmotic fluid absorption, and CFTR-mediated anion secretion is required for normal fluid secretion and tissue hydration. Recent data suggest that apical membrane CaCCs also play a role in normal hydration responses. In addition, a series of basolateral channels modify the driving forces for apical channel activity. Although maintenance of a normally hydrated surface layer in the airways depends on both secretory and absorptive processes, elegant studies by Shamsuddin and Quinton (34) question the hypothesis that cells can change from a secretory phenotype to an absorptive phenotype simply by activating different channels. These investigators suggested that, even within a confined area such as the small airways, there are cells dedicated to absorption and alternate cells that control secretion.

Calu-3 cells are a widely used model of airway serous cells that, in vivo, are undoubtedly secretory and one of the most important cell types involved in mucociliary clearance. Accordingly, these cells do not usually express ENaC, and our results are consistent with that premise. The presence of CFTR has long been established (34), and there have been suggestions of a Ca^{2+}-activated component to the anion secretion (11, 14, 15, 21, 22 25–27, 35, 38). Originally, this was envisioned as a stimulation of Ca^{2+}-activated K^+ channels on the basolateral membrane. Alternatively, others suggested that the oscillations were attributable to cAMP-stimulated K^+ channels.

K^+ channel activity is necessary to hyperpolarize the cells, thereby providing the driving force for anion efflux. In that regard, our data showing that serosal addition of $BaCl_2$ inhibits epinephrine-stimulated secretory current are consistent with the previous studies. However, in addition to the importance of Ca^{2+}-activated K^+ channels, more recent studies have demonstrated the presence of an apical CaCC. TMEM16A is highly expressed in secretory epithelial tissues, where it has been implicated to play a key role in Ca^{2+}-dependent Cl^- secretion (6, 11, 28, 29, 41). Fischer and colleagues (11) identified TMEM16A mRNA in both human serous cells and in the Calu-3 cell line. Functionally, ion channel studies of primary cultures from airways of TMEM16A knockout mice suggested that this CaCC is important in airway cells (28, 29). Isolated trachea from TMEM16a knockout mice exhibit reduced mucociliary clearance (26).

The Calu-3 cell response to epinephrine is complex and is composed of at least three components. First, there is a robust but very transient ion flux. This is followed by oscillating transport fluxes that dampen over time. Finally, there is a sustained level of transport that is fully inhibited by the nonspecific Cl^- channel blocker NPPB. Pretreatment of Calu-3 cells with either tannic acid (to inhibit TMEM16A) or CFTRinh172 (to inhibit CFTR) alone provides data suggesting that the initial transient peak is due to a combination of both CFTR and TMEM16A while the oscillatory peaks are dependent only on CFTR-mediated flux. The electrophysiological studies are complemented by assays showing that epinephrine stimulation resulted in both an increase in cAMP (Fig. 2) and an increase in intracellular Ca^{2+} (Fig. 10).

Pretreatment with a combination of CFTR inh172 and tannic acid indicates that basal as well as epinephrine-stimulated anion secretion is mediated by a combination of CFTR and TMEM16A. Interestingly, the inhibitors of each of the channels, when added together, cause an inhibition of all components of the transport response that is greater than the additive effects of each inhibitor alone. Finally, when we used a more specific inhibitor of TMEM16A, $T16A_{inh}$AO1, for the prein-

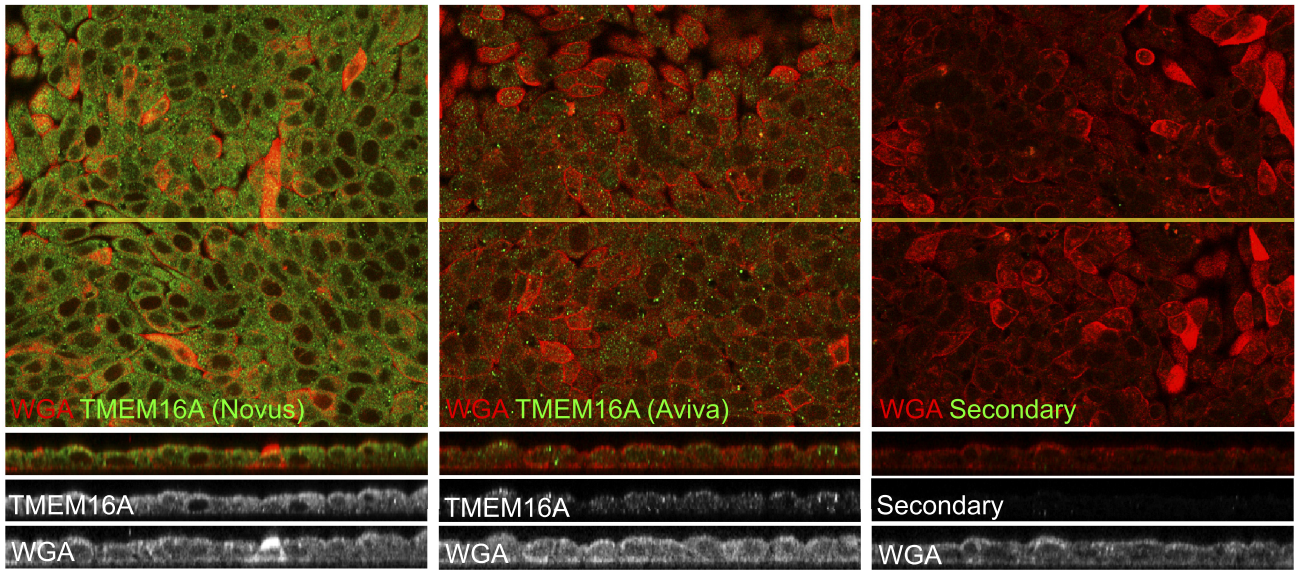

Fig. 9. Localization of TMEM16A in Calu-3 cells. Wheat germ agglutinin (WGA) 555 (red) stain was used to indicate the cell boundaries including the apical membrane. Orthogonal sections from the confocal image stack show apical localization in monolayers stained with the Novus and the Aviva anti-TMEM16A antibodies (green). *Insets*: from the yellow lines as indicated in left and center panels.

cubation step, all components of the epinephrine-stimulated current were substantially inhibited. These finding suggests complex and possibly compensatory interactions between the two channels.

Using agents that alter either intracellular cAMP or Ca^{2+} provides results that indicate that the control mechanisms may be more complex. Forskolin maximally increases intracellular cAMP by constitutively activating adenylyl cyclase. Forskolin

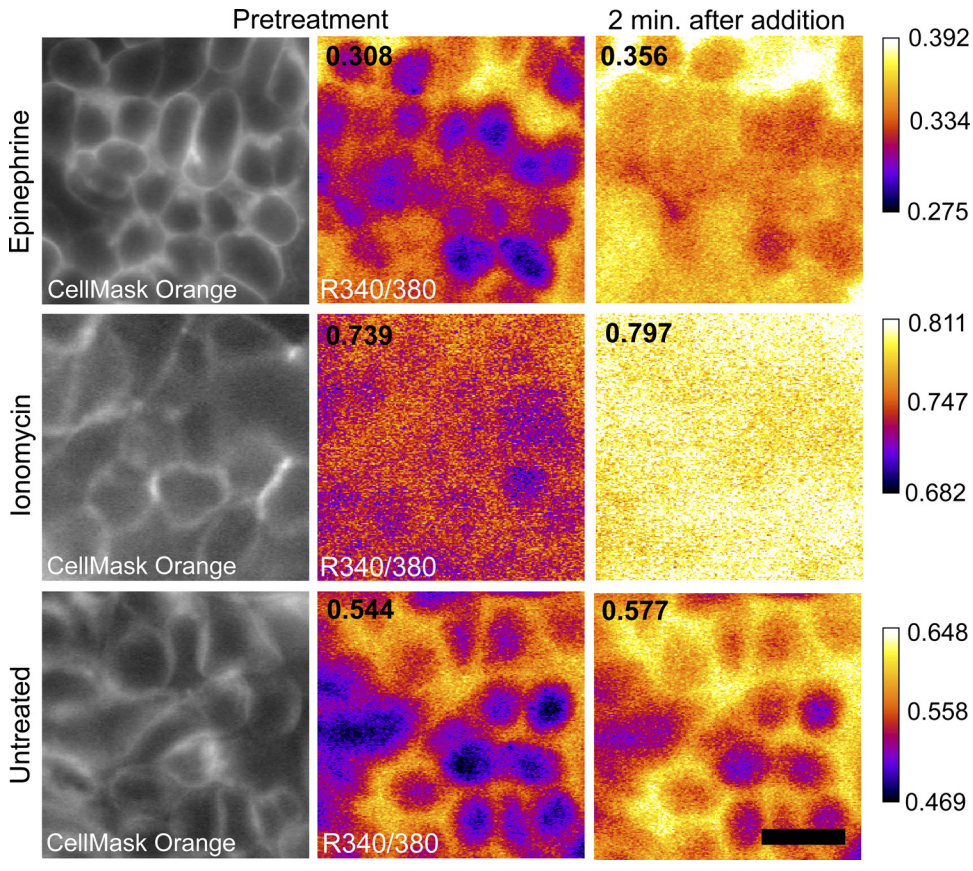

Fig. 10. Epinephrine-stimulated increases in intracellular calcium. Confluent monolayers were grown on Transwell membranes, incubated with fura 2 and CellMask orange, excised, mounted apical surface down onto the surface of a glass-bottom 35-mm dish, and imaged for 30 s with 2-s intervals before stimulation with epinephrine (10^{-6} M) or ionomycin (0.1 μM). Epinephrine induces an increase in Ca^{++} (*top*). *Middle* and *right*: The average R340/380 calculated for the whole field. *Left*: approximate cell volumes with CellMask Orange staining. Calibrations bars as indicated at right. Scale bar = 20 μm.

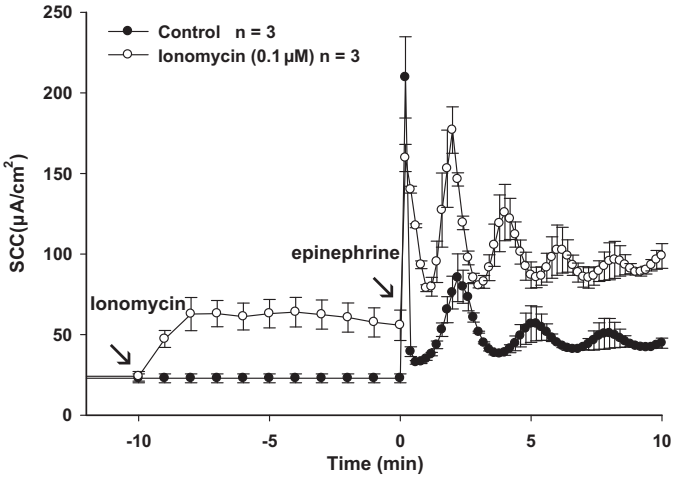

Fig. 11. Effect of ionomycin pretreatment on the epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable SCC. The graphs depict the continuous measurement of SCC, a measure of net ion transport of the cells. ●, Control; ○, cells were pretreated with ionomycin (0.1 μM) for 10 min. At $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of all experiments. The symbols on each plot denote the means of 3 experiments ± SE.

does, indeed, cause the oscillatory fluxes, but they are no larger than those seen with epinephrine stimulation, indicating that epinephrine maximally activates the cAMP pathway.

Addition of ionomycin to raise the intracellular Ca^{2+} concentration leads to a sustained increase in basal transport and renders the initial epinephrine-stimulated ion flux somewhat less transient. This is not surprising in that intracellular Ca^{2+} concentrations are mediated by organelle uptake, and any hormone-stimulated increase would be rapidly dissipated,

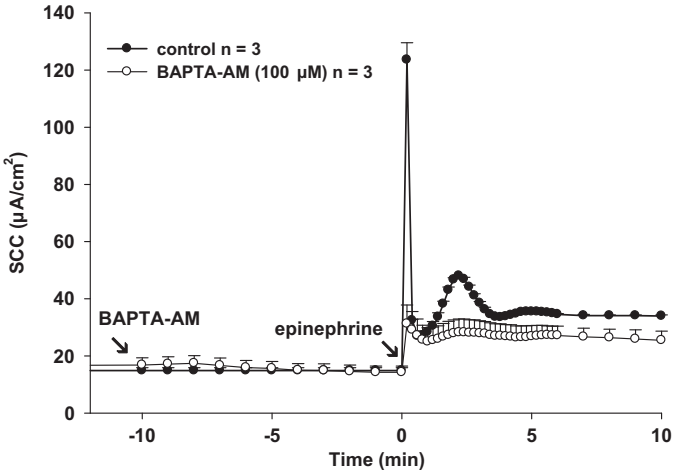

Fig. 12. Effect of 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) pretreatment on the epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14 days, excised, mounted in an Ussing chamber, and allowed to develop a stable basal SCC. The graphs depict the continuous measurement of SCC, a measure of net ion transport of the cells. ●, Control; ○, cells were pretreated with BAPTA-AM (100 μM) for 10 min. At $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of all experiments. The symbols on each plot denote the means of 3 experiments ± SE.

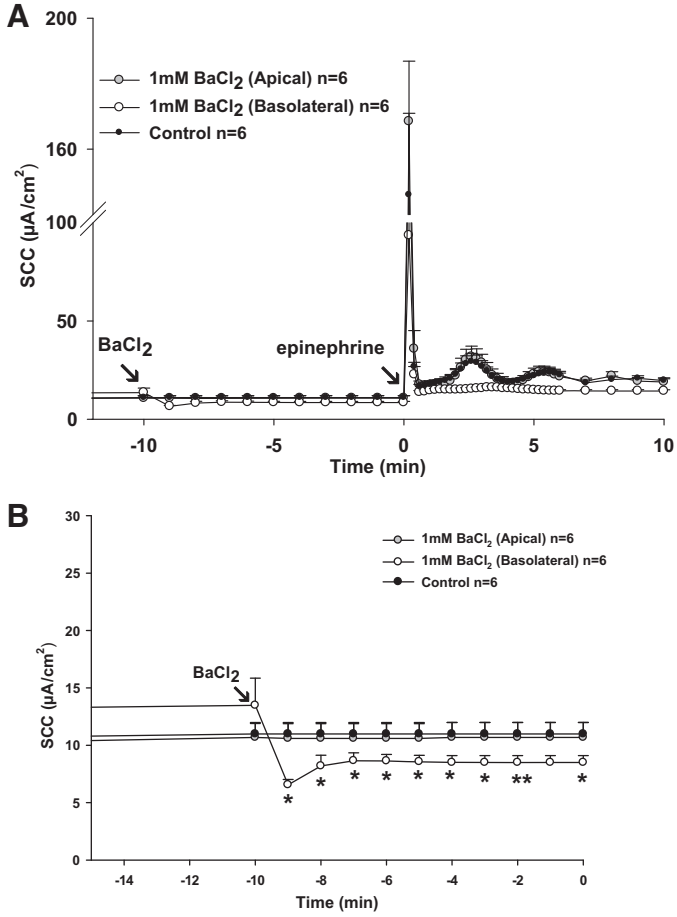

Fig. 13. Effect of $BaCl_2$ pretreatment on epinephrine-stimulated ion transport in the Calu-3 cell line. Confluent monolayers of Calu-3 cells were grown on permeable Transwell membranes for 14–16 days, excised, mounted in an Ussing chamber, and allowed to develop a stable SCC. The graphs depict the continuous measurement of SCC, a measure of net ion transport of the cells. Cells were pretreated with $BaCl_2$ for 10 min on either the apical or basolateral side of the cellular monolayer as indicated. *A*: at $t = 0$, epinephrine (10^{-6} M) was added to the serosal bathing media of all experiments. The symbols on each plot denote the means of 6 experiments ± SE. The values for the control and apically treated samples are superimposed for many of the time points. *B*: expanded view of the first 10 min of the $BaCl_2$ pretreatment. *Statistically lower than the control baseline, $P < 0.05$, 2-tailed Student's *t*-test.

whereas an external ionophore would lead to more sustained increases. What is rather unexpected is the increased periodicity of the CFTR-mediated oscillations, indicating that the intracellular Ca^{2+} concentration is modulating the apparent CFTR response. The importance of intracellular Ca^{2+} to the CFTR-mediated ion flux is further underscored by the finding that pretreatment with BAPTA-AM abolishes both the initial increase in anion transport and the oscillations but does not inhibit the sustained current.

In total, our results agree with other investigators who have used a variety of effectors to stimulate anion secretion in Calu-3 cells and documented the activity of both CFTR and a CaCC. Effectors that are specific for increasing intracellular Ca^{2+} such as carbachol, histamine, methacholine, and ATP as well as effectors such as forskolin that increase cAMP all increase Cl^-/HCO_3^- secretion (1, 11, 21, 22, 26, 36). In this regard, results in the Calu-3 cell line agree with both human and porcine primary cultures (11, 40). However, somewhat

unexpectedly, our results indicate that the natural hormone of the β_2-AR stimulates both cAMP- and Ca^{2+}-mediated increases in anion secretion, thereby potentially altering mucociliary clearance via multiple channels. In the entire respiratory system, it is clear that epinephrine can activate both secretion and absorption. This is made possible by the complex arrangement of cells that possess ENaC, CFTR, or CaCC as well as a range of channels, also present in specific cell types, that change the driving forces for ion flux, enabling the transepithelial transport of electrolytes and fluid on a cell-specific basis. Therefore, it follows that the clinical use of effectors such as the widely used β_2-AR agonists alters ion flux in different ways in each of the responsive cell types. Our results suggest that directed combination therapy might be more effective in severe respiratory pathologies. For example, epinephrine or long-acting β_2-AR agonists used as bronchodilators could be combined with inhibitors of ENaC, CFTR, or TMEM16A to specifically activate or inhibit fluid absorption or mucociliary clearance as required by the specific disease etiology.

In summary, our electrophysiological data as well as direct measurement of cAMP and confocal imaging showing an increase in intracellular Ca^{2+} indicate that epinephrine stimulates a secretory response that involves both CFTR and TMEM16A. Furthermore, the data agree with the premise that CFTR and TMEM16A are functionally linked, as suggested by Kunzelmann et al. (17). The simplest explanation of our findings is that the functional linkage is via changes in intracellular Ca^{2+}. In this regard, it is interesting that Billet and Hanrahan (4) have recently elucidated the pathway whereby Ca^{2+}-mobilizing cholinergic and purinergic responses activate CFTR (4). Clarification as to whether a similar mechanism may govern epinephrine-stimulated anion secretion in Calu-3 cells and the role of TMEM16A in potentiating this response awaits further experimentation.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: A.B., S.F., S.L., and S.W. performed experiments; A.B., S.F., S.L., S.W., and B.L.B.-Y. analyzed data; A.B., S.F., S.W., and B.L.B.-Y. prepared figures; A.B. and B.L.B.-Y. drafted manuscript; A.B., S.F., S.L., S.W., and B.L.B.-Y. approved final version of manuscript; S.F., S.W., and B.L.B.-Y. interpreted results of experiments; S.F., S.L., S.W., and B.L.B.-Y. edited and revised manuscript; B.L.B.-Y. conception and design of research.

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