Pharmacological modulation of ion transport across wild-type and ΔF508 CFTR-expressing human bronchial epithelia

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Devor, Daniel C., Robert J. Bridges, and Joseph M. Pilewski. Pharmacological modulation of ion transport across wild-type and ΔF508 CFTR-expressing human bronchial epithelia. Am J Physiol Cell Physiol 279: C461–C479, 2000.—Forskolin, UTP, 1-ethyl-2-benzimidazolinone (1-EBIO), NS004, 8-methoxypsoralen (Mexothaksalin; 8-MOP), and genistein were evaluated for their effects on ion transport across primary cultures of human bronchial epithelium (HBE) expressing wild-type (wt HBE) and ΔF508 (ΔF-HBE) cystic fibrosis transmembrane conductance regulator. In wt HBE, the baseline short-circuit current (Isc) averaged 27.0 ± 0.6 μA/cm² (n = 350). Amiloride reduced this Isc by 13.5 ± 0.5 μA/cm² (n = 317). In ΔF-HBE, baseline Isc was 33.8 ± 1.2 μA/cm² (n = 200), and amiloride reduced this by 29.6 ± 1.5 μA/cm² (n = 116), demonstrating the characteristic hyperabsorption of Na⁺ associated with cystic fibrosis (CF). In wt HBE, subsequent to amiloride, forskolin induced a sustained, bumetanide-sensitive Isc (ΔIsc = 8.4 ± 0.8 μA/cm²; n = 119). Addition of acetazolamide, 5-(N-ethyl-N-isopropyl)-amiloride, and serosal 4,4’-dinitrostilben-2,2'-disulfonic acid further reduced Isc suggesting forskolin also stimulates HCO₃⁻ secretion. This was confirmed by ion substitution studies. The forskolin-induced Isc was inhibited by 293B, Ba²⁺, clofibrate, and quinine, whereas charybdotoxin was without effect. In ΔF-HBE the forskolin Isc response was reduced to 1.2 ± 0.3 μA/cm² (n = 30). In wt HBE, mucosal UTP induced a transient increase in Isc (ΔIsc = 15.5 ± 1.1 μA/cm²; n = 44) followed by a sustained plateau, whereas in ΔF-HBE the increase in Isc was reduced to 5.8 ± 0.7 μA/cm² (n = 13). In wt HBE, 1-EBIO, NS004, 8-MOP, and genistein increased Isc by 11.6 ± 0.9 (n = 20), 10.8 ± 1.7 (n = 18), 10.0 ± 1.6 (n = 17), and 7.9 ± 0.8 μA/cm² (n = 17), respectively. In ΔF-HBE, 1-EBIO, NS004, and 8-MOP failed to stimulate Cl⁻ secretion. However, addition of NS004 subsequent to forskolin induced a sustained Cl⁻ secretory response (2.1 ± 0.3 μA/cm²; n = 21). In ΔF-HBE, genistein alone stimulated Cl⁻ secretion (2.5 ± 0.5 μA/cm²; n = 11). After incubation of ΔF-HBE at 26°C for 24 h, the responses to 1-EBIO, NS004, and genistein were all potentiated. 1-EBIO and genistein increased Na⁺ absorption across ΔF-HBE, whereas NS004 and 8-MOP had no effect. Finally, Ca²⁺-, but not cAMP-mediated agonists, stimulated K⁺ secretion across both wt HBE and ΔF-HBE in a glibenclamide-dependent fashion. Our results demonstrate that pharmacological agents directed at both basolateral K⁺ and apical Cl⁻ conductances directly modulate Cl⁻ secretion across HBE, indicating they may be useful in ameliorating the ion transport defect associated with CF.

The hallmark ion transport defects in cystic fibrosis (CF) are a diminished or absent Cl⁻ secretory response to cAMP-mediated agonists and Na⁺ hyperabsorption. The most common mutation in the CF transmembrane conductance regulator (CFTR) gene product, a deletion of phenylalanine at amino acid 508 (ΔF508), is particularly insidious, in that it leads to both a defect in the trafficking of the mutant protein to the apical membrane (55) as well as a channel that exhibits defective gating (1, 44). Pharmacologically, there are theoretically several means to ameliorate this primary ion transport defect. First, one can correct the trafficking defect associated with ΔF508 CFTR (5). This approach was highlighted by Denning et al. (9) who demonstrated that decreasing the temperature at which ΔF508 CFTR-expressing cells are grown increases the trafficking of the mutant protein to the plasma membrane.

A second strategy is to develop agents capable of directly interacting with any ΔF508 CFTR expressed in the apical membrane. Highlighting this possibility, Kalin et al. (31) recently demonstrated that ΔF508 CFTR can be expressed at the apical membrane of CF airway. The benzimidazolone, 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazole-2-one (NS004) was the first compound shown to activate both wild-type and ΔF508 CFTR in excised patch-clamp recordings (23). However, we demonstrated that neither NS004 nor its structurally related analog, NS1619, stimulated Cl⁻ secretion in either the T84 cell line or in primary cultures of murine tracheal epithelium (MTE), despite the fact that these compounds increased apical membrane Cl⁻ conductance (13). Nguyen et al. (41) first demonstrated that the flavones quercetin and kaempferol stimulated Cl⁻ secretion across the T84 model secretory epithelium in a cAMP-independent manner. Subsequently, it has been shown

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that the related compound, genistein, also stimulates CI− secretion (28). More recent evidence indicates that genistein directly interacts with CFTR to increase the open probability of the channel (25, 54).

Finally, one can bypass the CFTR defect altogether by modulating the activity of alternative ion conductive pathways. For example, increasing intracellular Ca2+ has been shown to stimulate Cl− secretion across CF airway (57). Indeed, Mason et al. (37) demonstrated that the Ca2+-dependent agonist, UTP, acting at P2Y2 receptors, stimulates Cl− secretion across CF tracheal epithelium. We previously characterized the basolateral membrane K+ channel activated by Ca2+-mediated agonists (KCa) in colonic and airway epithelia (10, 16). We demonstrated that direct pharmacological activation of KCa by the benzimidazolone, 1-ethyl-2-benzimidazolone (1-EBIO), resulted in the stimulation of Cl− secretion across T84 and Calu-3 cells as well as primary cultures of MTE (13, 15). These results suggest that basolateral K+ channels may represent unique pharmacological targets for CF therapy (13, 15, 16).

Although 1-EBIO, genistein, 8-methoxypsoralen (Methoxsalen; 8-MOP), and NS004 have been shown to stimulate Cl− secretion across T84 cells and MTE, the effects of these compounds on CI− secretion across human airway have not been evaluated. Therefore, we determined the effects of these agonists on primary cultures of human bronchial epithelia (HBE) expressing wild-type (wt HBE) or ΔF508 (ΔF-HBE) CFTR. We demonstrate that 1-EBIO, NS004, 8-MOP, and genistein stimulate a sustained Cl− secretory response in wt HBE. In ΔF-HBE both NS004 and genistein stimulate a small Cl− secretory response, whereas 1-EBIO and 8-MOP do not. Additionally, following incubation of the cells at 26°C, the responses to 1-EBIO, NS004, and genistein are all potentiated. These results indicate that an apical membrane Cl− conductance, perhaps ΔF508 CFTR, is expressed and can be pharmacologically modulated in ΔF-HBE. Further, these results demonstrate that, despite the low levels of expression of CFTR in native tissue, pharmacological agents directed at either apical Cl− or basolateral K+ channels are capable of modulating Cl− secretion, supporting the notion that they may be therapeutically useful for CF.

METHODS

Primary Cultures of HBE

HBE was obtained from excess pathological tissue remaining after lung transplantation under a protocol approved by the University of Pittsburgh Investigational Review Board. Tissue expressing wt CFTR was obtained following lung transplantation for a variety of pathological conditions including bronchiectasis, emphysema, primary pulmonary hypertension, pulmonary fibrosis, and α1-antitrypsin deficiency. Except for one tissue sample that was compound heterozygote (ΔF508:2789 +5G→A), all CF tissues employed in this study were homozygous for the ΔF508 CFTR mutation by allele-specific hybridization (performed at Genzyme, Framingham, MA). All cells were isolated from second through sixth generation bronchi in both wt CFTR-expressing and CF HBE. The bronchi were incubated overnight at 4°C in MEM containing 0.1% protease XIV, 0.01% deoxyribonuclease, and 1% FBS. The epithelial cells were removed from the underlying musculature by blunt dissection, isolated by centrifugation, and washed in MEM containing 5% FBS. After centrifugation, the cells were resuspended in bronchial epithelial growth media (BEGM; catalog no. CC-3170; Clonetics, San Diego, CA). The cells were then plated into HPC-treated t-25 tissue culture flasks. On reaching 80–90% confluence, the cells were trypsinized (0.1%), resuspended in MEM plus 5% FBS, and seeded onto HPC-coated Costar Transwell filters (0.33 cm2) at a density of ~2 × 106/cm2. After 24 h the media were changed to DMEM/F-12 (1:1) plus 2% Ultroser G (BioSepra, Cedex, France), and an air interface at the apical membrane was established. The media bathing the basolateral surface were changed every 48 h. Measurements of short-circuit current (Isc) were performed after ~10–20 additional days in culture.

Isc Measurements

Costar Transwell cell culture inserts were mounted in an Ussing chamber (Jim’s Instruments, Iowa City, IA), and the monolayers were continuously short circuited (University of Iowa, Department of Bioengineering). Transepithelial resistance was measured by periodically applying a 5-mV pulse and the resistance calculated using Ohm’s law. The bath solution contained (in mM) 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.8 K2HPO4, 1.2 MgCl2, 1.2 CaCl2, and 10 glucose. The pH of this solution was 7.4 when gassed with a mixture of 95% O2-5% CO2 at 37°C. In zero Cl− solutions, all Cl− was replaced with gluconate. In HCO3−-free solutions the NaHCO3 was replaced by 20 mM HEPES and the pH adjusted to 7.4 with NaOH. The effects of 1-EBIO on basolateral membrane K+ currents (I(K)) were assessed following permeabilization of the apical membrane with nystatin (180 μg/ml) for 15–30 min and establishment of a mucosa-to-serosa K+ concentration gradient. For measurements of K+ mucosal NaCl was replaced by equimolar potassium gluconate, while serosal NaCl was substituted with equimolar sodium gluconate (15). The CaCl2 was increased to 4 mM to compensate for the Ca2+-buffering capacity of the gluconate anion. For measurement of K+ secretory currents, serosal NaCl was replaced by equimolar potassium gluconate, while mucosal NaCl was substituted with equimolar sodium gluconate because 1-EBIO, NS004, genistein, 8-MOP, acetazolamide, and forskolin are lipophilic in nature, they will readily cross between apical and basolateral compartments; thus these compounds were added to both sides of the monolayer at the indicated concentration. UTP and amiloride were added only to the mucosal bathing solution. Bumetanide and 4,4’-dinitrostilben-2,2’-disulfonic acid (DNDS) were added only to the serosal bathing solution. Changes in Isc were calculated as the difference in current between either the peak or sustained phase of the response and their respective baseline values.

Chemicals

NS004 was a generous gift from Dr. Soren Peter-Olesen (Neurosearch, Glostrup, Denmark). 293B was a generous gift from Dr. Rainer Greger (Albert-Ludwigs-Universitat, Freiberg, Germany), and nystatin was a generous gift from Dr. S. Lucania (Bristol Meyers-Squibb). 1-EBIO was obtained from Aldrich Chemical; genistein was obtained from Indofine Chemical (Somerville, NJ); UTP was obtained from Calbiochem (La Jolla, CA); bumetanide, quinine, 8-MOP, and.
forskolin were obtained from Sigma Chemical (St. Louis, MO). 5-α-ethyl-N-isopropylamiloride (EIPA) and chlordinium were obtained from RBI (Natick, MA). DNDS was obtained from Pfaltz and Bauer (Westbury, CT). Charybdoxin (CTX) was obtained from Accurate Chemical and Scientific (Westbury, NY). All compounds, prepared in either DMSO or ethanol, were made as ≥1,000-fold stock solutions. Neither DMSO nor ethanol alone at ≤0.1% had any effect on $I_{sc}$. Cell culture medium was obtained from GIBCO unless otherwise noted above.

**Data Analysis**

All data are presented as means ± SE, where $n$ indicates the number of experiments. Apparent inhibitory ($K_i$) and stimulatory ($K_e$) constants were obtained using nonlinear curve-fitting routines in SigmaPlot (Jandel Scientific, San Rafael, CA). Statistical analysis was performed using Student's $t$-test. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

In total, we evaluated 350 wt CFTR-expressing HBE monolayers from 40 patients under short-circuit conditions in symmetrical standard bath solution. The baseline $I_{sc}$ averaged 27.0 ± 0.6 μA/cm² with a transepithelial potential difference (PD), and resistance ($R_{te}$) of -15.6 ± 0.5 mV and 646 ± 18 Ω cm², respectively. Addition of amiloride (10 μM) to the mucosal chamber reduced $I_{sc}$ an average of 13.5 ± 0.5 μA/cm² to a new plateau value of 13.5 ± 0.4 μA/cm² ($n = 317$). In contrast, in 200 monolayers, from 16 patients homozygous for the ΔF508 CFTR mutation (ΔF-HBE), the baseline $I_{sc}$ averaged 33.8 ± 1.2 μA/cm² with a PD and $R_{te}$ of -17.5 ± 0.6 mV and 568 ± 24 Ω cm², respectively. Addition of amiloride to these ΔF-HBE reduced $I_{sc}$ by 29.6 ± 1.5 μA/cm² to a new plateau value of 5.1 ± 0.2 μA/cm² ($n = 116$). Thus amiloride reduces $I_{sc}$ by 87% in ΔF-HBE, demonstrating a significant Na⁺ hyperabsorption in our ΔF-HBE cultures ($P < 0.0001$). Our data on wt CFTR-expressing HBE are presented first, and data on ΔF-HBE are presented in later sections.

**Effect of Forskolin on Ion Transport Across HBE**

The effect of forskolin (10 μM), subsequent to amiloride, on ion transport across wt HBE is shown for one monolayer in Fig. 1A. Forskolin induced an initial peak in $I_{sc}$, followed by a sustained plateau (see also Figs. 2 and 6). In 119 monolayers forskolin increased $I_{sc}$ from an amiloride-inhibited plateau of 16.0 ± 0.7 to 30.0 ± 1.4 μA/cm² followed by a decline to a stable plateau of 24.4 ± 1.1 μA/cm². As shown in Fig. 1A, the Na⁺-K⁺-2Cl⁻ cotransporter inhibitor bumetanide reduced $I_{sc}$ to below the $I_{sc}$ level observed in the presence of amiloride. In 13 monolayers amiloride reduced $I_{sc}$ to 21.1 ± 1.9 μA/cm². Forskolin induced a sustained increase in $I_{sc}$ to 43.1 ± 15.9 μA/cm², and this was reduced to 12.3 ± 1.5 μA/cm² by bumetanide.

Our results suggest that a portion of the amiloride-insensitive $I_{sc}$ is due to Cl⁻ secretion. However, the $I_{sc}$ does not approach zero in the presence of the combination of amiloride plus bumetanide, suggesting an additional ongoing active transport process. Smith and Welsh (47) previously demonstrated that canine trachea is capable of secreting HCO₃⁻ in response to cAMP-mediated agonists and also demonstrated that primary cultures of human airway respond to forskolin in Cl⁻-free solutions, suggesting a HCO₃⁻ secretory process. Also, we recently demonstrated that Calu-3 cells secrete HCO₃⁻ in response to elevated cAMP (16). Thus, in an initial attempt to determine whether a portion of the amiloride-insensitive $I_{sc}$ observed may be due to HCO₃⁻ secretion, we utilized a combination of the carbonic anhydrase inhibitor acetazolamide (100 μM) and the Na⁺/H⁺ exchange inhibitor EIPA (5 μM).

As shown in Fig. 1A, acetazolamide plus EIPA reduced $I_{sc}$ an additional 3.6 ± 0.4 μA/cm² ($n = 4$), suggesting this basal $I_{sc}$ may be due to HCO₃⁻ secretion. The magnitude of this inhibition is similar to what was reported by Smith and Welsh (47) using acetazolamide (1 mM) and serosal amiloride (1 mM) in canine trachea.

To further evaluate the possibility that forskolin is stimulating HCO₃⁻ secretion across wt HBE, we performed ion substitution experiments in which Cl⁻ or both Cl⁻ and HCO₃⁻ were removed from both the mucosal and serosal solutions (see Methods). As shown in Fig. 1B, in the absence of Cl⁻, forskolin stimulated a bumetanide-insensitive increase in $I_{sc}$ that was partially inhibited by acetazolamide. In 11 experiments forskolin increased $I_{sc}$ an average of 6.3 ± 1.3 μA/cm² in the absence of Cl⁻. By comparison, in the absence of both Cl⁻ and HCO₃⁻, forskolin increased $I_{sc}$ by only 0.9 ± 0.4 μA/cm² ($n = 6$). These experiments demonstrate that forskolin stimulates a transepithelial current response that is dependent on HCO₃⁻ in the bathing solution.

We recently demonstrated that the human airway cell line Calu-3 secretes HCO₃⁻ by a Na⁺-dependent mechanism in response to forskolin and that this could be inhibited by serosal DNDS (16). High concentrations of DNDS (1–3 mM) have been shown to inhibit the Na⁺-HCO₃⁻ cotransporter (4, 56), suggesting that this cotransporter was responsible for HCO₃⁻ entry across the serosal membrane of Calu-3 cells [ribonucleoside diphosphate reduction protection assays confirm expression of a Na⁺-HCO₃⁻ cotransporter (NBC) in Calu-3 cells as well as HBE (Gangopadhyay NN and Bridges RJ, unpublished observations)]. We therefore determined whether DNDS would similarly inhibit forskolin-mediated anion transport across wt HBE. As shown in Fig. 1C, in the absence of mucosal and serosal Cl⁻, serosal DNDS (3 mM) partially inhibited the forskolin-induced $I_{sc}$, and this was further reduced by the addition of acetazolamide. In five monolayers, forskolin increased $I_{sc}$ from 3.4 ± 0.3 to 7.4 ± 1.0 μA/cm², and this was reduced to 5.4 ± 0.8 and 3.7 ± 0.5 μA/cm² by DNDS and acetazolamide, respectively. These results suggest that a DNDS-sensitive Na⁺-HCO₃⁻ cotransporter is partially responsible for serosal HCO₃⁻ entry in HBE, a
mechanism similar to that which we described in Calu-3 cells (16).

Although it is likely that CFTR represents the apical membrane Cl\(^{-}\) channel activated by forskolin, the basolateral membrane K\(^{+}\) channels involved in maintaining the driving force for Cl\(^{-}\) secretion have received little attention. Therefore, we evaluated the effect of several known K\(^{+}\) channel blockers on the forskolin-stimulated \(I_{sc}\). We previously demonstrated that CTX inhibits Ca\(^{2+}\) but not cAMP-dependent Cl\(^{-}\) secretion across T84 cells (15). Similarly, in wt HBE cells, CTX (50 nM) had no effect on forskolin-stimulated Cl\(^{-}\) secretion (Fig. 2). Similar results were obtained in six additional experiments. Lohrmann et al. (34) first described the chromanol, 293B, as a highly selective inhibitor of the basolateral membrane cAMP-dependent K\(^{+}\) channel. We determined the effect of 293B (100 \(\mu\)M) on the forskolin-stimulated \(I_{sc}\) in wt HBE cells.

Fig. 1. Effect of forskolin on ion transport across wild-type (wt) cystic fibrosis transmembrane conductance regulator (CFTR)-expressing human bronchial epithelia (wt HBE). A: in the presence of mucosal and serosal NaCl-containing solutions, forskolin (Forsk; 10 \(\mu\)M) stimulated a sustained increase in short-circuit current (\(I_{sc}\)) subsequent to amiloride (Amil; 10 \(\mu\)M). Bumetanide (Bumet; 20 \(\mu\)M) reduced \(I_{sc}\) to below the amiloride-dependent baseline, and this \(I_{sc}\) was further reduced by the addition of the carbonic anhydrase inhibitor acetazolamide (Acetazol; 100 \(\mu\)M) and the Na\(^{+}/H^{+}\) exchange inhibitor 5-(N-ethyl-N-isopropyl)-amiloride (EIPA; 5 \(\mu\)M, serosal). B: in the absence of mucosal and serosal Cl\(^{-}\), forskolin induced a sustained, bumetanide-insensitive \(I_{sc}\) that was inhibited by the subsequent addition of acetazolamide. C: in the absence of mucosal and serosal Cl\(^{-}\), serosal 4,4'-dinitrostilben-2,2'-disulfonic acid (DNDS; 3 mM) inhibited the forskolin-dependent increase in \(I_{sc}\). Dashed lines, zero current level.

Fig. 2. Effect of K\(^{+}\) channel blockers on the forskolin-dependent increase in \(I_{sc}\) across wt CFTR-expressing HBE. Subsequent to amiloride (10 \(\mu\)M), forskolin induced a sustained increase in \(I_{sc}\) that was insensitive to block by charybdotoxin (CTX; 50 nM, serosal). However, the subsequent addition of both 293B (100 \(\mu\)M, serosal) and Ba\(^{2+}\) (5 mM, serosal) inhibited this \(I_{sc}\). Bumetanide (20 \(\mu\)M) further reduced \(I_{sc}\). Dashed line, zero current level.
In 12 monolayers, forskolin increased $I_{sc}$ from $17.1 \pm 2.8 \mu A/cm^2$ to a sustained value of $23.4 \pm 3.4 \mu A/cm^2$, and this was reduced by 67% to $19.2 \pm 2.7 \mu A/cm^2$ by 293B ($P < 0.001$). These results demonstrate that, similar to colonic epithelia (15, 34), human airway expresses a 293B-sensitive $K^+$ conductance.

Although it is clear that 293B inhibits a significant portion (67%) of the cAMP-mediated $I_{sc}$, a large 293B-independent $I_{sc}$ remains (Fig. 2). Our results above (Fig. 1) suggest that a portion of this current is due to Cl$^-$ secretion based on its bumetanide sensitivity and requirement for Cl$^-$.

Thus we determined whether other nonselective $K^+$ channel blockers would further inhibit this basal Cl$^-$ secretion. Subsequent to 293B, addition of Ba$^{2+}$ (5 mM) reduced $I_{sc}$ to a sustained value of $8.5 \pm 1.5 \mu A/cm^2$ ($n = 10$; Fig. 2), with bumetanide further reducing $I_{sc}$ to $3.7 \pm 0.5 \mu A/cm^2$ ($n = 10$). Subsequent to 293B, quinine further reduced $I_{sc}$ from $9.8 \pm 0.6$ to $4.3 \pm 0.8 \mu A/cm^2$ ($n = 4$). Finally, clofilium (100 $\mu M$) inhibited both the forskolin-dependent and -independent current, reducing $I_{sc}$ from $42.0 \pm 12.5$ to $4.7 \pm 1.2 \mu A/cm^2$ ($n = 4$). These results suggest that there is a Ba$^{2+}$-, clofilium-, and quinine-sensitive basolateral $K^+$ conductance that underlies the bumetanide-sensitive Cl$^-$ secretion induced by amiloride and forskolin.

Effect of the CFTR Openers NS004, 8-MOP, and Genistein on Cl$^-$ Secretion Across wt HBE

NS004. Gribkoff et al. (23) characterized NS004 as the first known opener of both wt and ΔF508 CFTR in the Xenopus oocyte heterologous expression system. Subsequently, we demonstrated that, although NS004 increased apical membrane Cl$^-$ conductance in the T84 cell line, it failed to induce a Cl$^-$ secretory response (13). Also, NS004 failed to stimulate Cl$^-$ secretion in primary cultures of MTE (13). In contrast to these results, NS004 (10 $\mu M$) stimulated a sustained, bumetanide-sensitive increase in $I_{sc}$ across wt HBE, subsequent to amiloride (Fig. 3A). This concentration of NS004 was chosen based on both the $K_s$ (see below) for NS004 as well as our previous studies, demonstrating that higher concentrations of NS004 disrupted epithe-

![Fig. 3](http://ajpcell.physiology.org/)
lial integrity in T84 cells (13). In 18 experiments, NS004 increased $I_{sc}$ by 10.8 ± 1.7 μA/cm², from 13.4 ± 0.9 to 24.2 ± 2.1 μA/cm². In eight of these experiments, the subsequent addition of bumetanide reduced $I_{sc}$ to 10.4 ± 0.6 μA/cm². In nine additional monolayers, a complete concentration-response curve for NS004 was generated. These data were fitted to a Michaelis-Menten function having an apparent $K_m$ of 1.2 ± 0.3 μM (Fig. 3B).

One explanation for the divergent results between T84 cells (13) and HBE is a potential difference in the ability of NS004 to modulate basolateral membrane K⁺ channels in HBE; NS004 was initially characterized as an opener of CTX-sensitive maxi-K channels (43). However, CTX (50 nM) failed to inhibit the NS004-induced $I_{sc}$ in HBE, arguing against a role for maxi-K channels in this Cl⁻ secretory response (data not shown). Also, NS004 failed to modulate Cl⁻ secretion in MTE subsequent to amiloride (13), suggesting that the hyperpolarization of the apical membrane induced by amiloride (58) cannot fully explain the difference between MTE and wt HBE. Based on our results in T84 cells, we proposed that the basolateral membrane K⁺ conductance ($G_K$) was rate limiting for NS004-mediated Cl⁻ secretion across colonic epithelia (13). Thus an alternative possibility is that CFTR is rate limiting in wt HBE, rather than $G_K$. To test this hypothesis we determined whether prior addition of Ba²⁺ would inhibit the NS004-induced Cl⁻ secretory response as predicted if CFTR is rate limiting. As shown in Fig. 3C, addition of Ba²⁺ inhibited the basal, amiloride-insensitive $I_{sc}$. The subsequent addition of NS004 (10 μM) increased $I_{sc}$ by 5.5 ± 1.0 μA/cm² ($n = 9$), which is significantly less than in the absence of Ba²⁺ ($P < 0.05$). Addition of 1-EBIO (300 μM), an activator of the Ca²⁺-dependent, CTX-sensitive K⁺ channel, resulted in a sustained Cl⁻ secretory response ($\Delta I_{sc} = 11.1 ± 3.0$ μA/cm²; $n = 9$) that was inhibited by CTX (50 nM; $\Delta I_{sc} = 11.1 ± 5.3$ μA/cm²; $n = 9$). We previously demonstrated that the 1-EBIO-activated K⁺ channel in T84 cells is sensitive to block by CTX but insensitive to Ba²⁺ (10). These results demonstrate that NS004 is capable of stimulating Cl⁻ secretion across wt HBE and suggest that the activation of CFTR is the rate-limiting step for Cl⁻ secretion across HBE expressing wt CFTR.

We previously demonstrated that NS004 failed to increase Cl⁻ secretion, subsequent to forskolin, in T84 cells (13). Similarly, in wt HBE, NS004 (10 μM) increased $I_{sc}$ by only 1.2 ± 0.3 μA/cm² ($n = 7$) after addition of forskolin (10 μM). Also, forskolin increased $I_{sc}$ by only 0.6 ± 0.4 μA/cm² ($n = 6$) subsequent to NS004. These results indicate that both forskolin and NS004 are capable of increasing apical Cl⁻ conductance to a level where it is no longer rate limiting for Cl⁻ secretion.

Although our results demonstrate that NS004 is capable of stimulating Cl⁻ secretion in the presence of a favorable driving force induced by amiloride, it is important to determine whether pharmacological agents are capable of stimulating Cl⁻ secretion in the absence of this enhanced driving force. As shown be-

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**Psoralsens.** We previously demonstrated that the psoralsens increase apical membrane Cl⁻ conductance and stimulate Cl⁻ secretion across both T84 cells and MTE (14). Similar to our results with NS004, stimulation of transepithelial Cl⁻ secretion required the addition of a K⁺ channel agonist such as 1-EBIO or carbachol (14). Based on blocker pharmacology, we proposed that the psoralsens stimulated Cl⁻ secretion via an activation of CFTR (14). We evaluated the effect of 8-MOP in wt HBE following inhibition of basal Na⁺ absorption with amiloride. The maximal effective concentration of 8-MOP for increasing apical $G_{Cl}$ in T84 cells was 30 μM. To directly compare the effects of NS004 and 8-MOP, we chose to utilize 10 μM 8-MOP for our HBE studies. As shown in Fig. 4, 8-MOP induced a sustained, bumetanide-sensitive increase in Cl⁻ secretion across wt HBE. In five monolayers, 8-MOP increased $I_{sc}$ from 10.0 ± 2.3 to 20.0 ± 0.4 μA/cm². Addition of bumetanide reduced this current to 9.7 ± 1.4 μA/cm². These results confirm that, following hyperpolarization of the apical membrane with amiloride, Cl⁻ channel agonists are capable of stimulating Cl⁻ secretion.
across wt HBE. In addition, we determined the effect of 8-MOP on $I_{sc}$ in the absence of amiloride. 8-MOP had no significant effect on Na$^+$ transport (see Effect of NS004, 8-MOP, 1-EBIO, and Genistein on Na$^+$ Absorption Across ΔF-HBE), suggesting any increase in $I_{sc}$ was due to anion secretion. Similar to NS004, 8-MOP induced a small, albeit significant increase in $I_{sc}$ of $3.7 \pm 1.3 \mu A/cm^2$ ($n = 5$, $P < 0.05$) in the absence of amiloride.

**Genistein.** Genistein has been shown to stimulate Cl$^-$ secretion across both T84 (28) and Calu-3 (26) cells, and more recently has been demonstrated to directly activate both wt and ΔF508 CFTR in excised patches (25, 54). We therefore determined whether genistein would stimulate transepithelial Cl$^-$ secretion across wt CFTR-expressing HBE. As shown in Fig. 5A, subsequent to amiloride, genistein (50 μM) induced an initial peak increase in $I_{sc}$ followed by a sustained, bumetanide-sensitive plateau. This concentration of genistein was chosen based on previous studies (27, 45) as well as on the $K_s$ determined in our own studies (see below). In 17 monolayers, genistein induced an initial peak increase in $I_{sc}$ from $11.0 \pm 1.3$ to $18.9 \pm 1.2 \mu A/cm^2$ followed by a sustained plateau at $16.6 \pm 1.3 \mu A/cm^2$. The addition of bumetanide in nine of these experiments reduced $I_{sc}$ to $5.4 \pm 0.7 \mu A/cm^2$. In an additional six monolayers a complete concentration-response relationship was generated for genistein and the data fitted to a Michaelis-Menten function having an apparent $K_s$ of $1.9 \pm 0.4 \mu M$ (Fig. 5B). We were unable to evaluate the effects of genistein on anion secretion in the absence of amiloride as we have demonstrated that genistein stimulates a significant increase in Na$^+$ absorption across HBE (12) (see Effect of NS004, 8-MOP, 1-EBIO, and Genistein on Na$^+$ Absorption Across ΔF-HBE). Thus changes in $I_{sc}$ could not be reliably attributed to either cation absorption or anion secretion.

We next determined whether genistein and forskolin would induce an additive increase in $I_{sc}$. However, similar to NS004, genistein (50 μM) increased $I_{sc}$ by only $1.7 \pm 0.2 \mu A/cm^2$ ($n = 10$) subsequent to forskolin. Surprisingly, addition of forskolin (10 μM) after genistein resulted in a small, but significant decrease in $I_{sc}$ of $1.6 \pm 0.6 \mu A/cm^2$ ($n = 8$; $P < 0.05$). Again, this lack of an additive response is consistent with both

![Fig. 5. Effect of genistein (50 μM) on ion transport across wt CFTR-expressing HBE. A: in the presence of mucosal and serosal NaCl, genistein induced a sustained increase in $I_{sc}$ that was inhibited by bumetanide (20 μM). B: concentration-response curve for the genistein-induced $I_{sc}$ response in wt HBE, subsequent to amiloride. Data were fitted to a Michaelis-Menten function with an apparent $K_s$ of $1.9 \pm 0.4 \mu M$. C: in the absence of mucosal and serosal Cl$^-$, genistein stimu- lated a sustained increase in $I_{sc}$ that was inhibited by both serosal DNDS (3 mM) and acetazolamide (100 μM). Dashed lines, zero current level.](http://ajpcell.physiology.org/content/early/2017/06/09/ajpcell.2017.006214/F5.2)
genistein and forskolin activating CFTR such that the activation of an apical Cl− conductance is no longer rate limiting to Cl− secretion after the addition of one of these agents.

We next determined whether pharmacological activation of CFTR by genistein would stimulate $I_{sc}$ in the absence of Cl−, indicative of HCO3− secretion. As shown in Fig. 5C, in the absence of mucosal and serosal Cl− (see METHODS), genistein (50 μM) increased $I_{sc}$ in a serosal DNDS (3 mM)- and acetazolamide (100 μM)-sensitive manner, consistent with our results above with forskolin. In three experiments, genistein increased $I_{sc}$ from 3.1 ± 0.4 to 7.0 ± 1.1 μA/cm² ($P < 0.05$), and this was reduced to 5.4 ± 1.4 and 4.5 ± 1.6 μA/cm² by DNDS and acetazolamide, respectively ($P < 0.05$). These results suggest that, similar to forskolin and NS004, genistein induces both Cl− and HCO3− secretion across wt HBE.

Effect of the Ca2+-Dependent K+ Channel Opener 1-EBIO on Cl− Secretion Across wt HBE

We previously demonstrated that 1-EBIO directly activates KCa channels in excised membrane patches and stimulates a sustained Cl− secretory response in both T84 and Calu-3 cells with a $K_{1/2}$ of ~500 μM (13, 15, 16). Similarly, in wt HBE, subsequent to amiloride, 1-EBIO (1 mM) induced a sustained increase in $I_{sc}$ (Fig. 6A). In contrast to our results with NS004 and genistein, the subsequent addition of forskolin (10 μM) resulted in a further increase in $I_{sc}$. Addition of the KCa channel blocker CTX (50 nM) inhibited $I_{sc}$. As shown above, CTX has no effect on the forskolin-induced $I_{sc}$, indicating that this inhibition is due to the 1-EBIO-dependent activation of a CTX-sensitive K+ channel. Addition of bumetanide resulted in a further inhibition of $I_{sc}$. Similarly, addition of 1-EBIO subsequent to forskolin induced a further, CTX-sensitive increase in $I_{sc}$ (Fig. 6B). In 20 experiments, 1-EBIO (1 mM) increased $I_{sc}$ from 11.3 ± 0.8 to 22.9 ± 1.2 μA/cm². In eight of these experiments, addition of forskolin further increased $I_{sc}$ by 3.4 ± 1.3 μA/cm² ($P < 0.05$), and this was reduced by CTX (50 nM) and bumetanide to 19.7 ± 0.8 and 9.9 ± 0.4 μA/cm², respectively. In an additional seven experiments, forskolin increased $I_{sc}$ from 11.2 ± 1.0 to 19.8 ± 0.7 μA/cm², and 1-EBIO (1 mM) further increased $I_{sc}$ by 6.5 ± 1.2 μA/cm². The subsequent addition of CTX (50 nM) and bumetanide reduced $I_{sc}$ to 19.3 ± 0.9 and 9.9 ± 0.5 μA/cm², respectively. These results demonstrate that pharmacological activation of basolateral membrane K+ channels is an alternative strategy to stimulate Cl− secretion across human bronchial epithelia.

Our results above suggest that forskolin, as well as the Cl− channel openers, genistein and NS004, stimulate HCO3− secretion across wt HBE, in addition to their effects on Cl− secretion. Because 1-EBIO activates both CFTR and KCa (15), we determined whether 1-EBIO would similarly stimulate $I_{sc}$ in a Cl−-independent manner, suggestive of HCO3− transport. In the absence of mucosal and serosal Cl−, 1-EBIO (1 mM) increased $I_{sc}$ from 4.4 ± 0.3 to 8.1 ± 1.1 μA/cm² (n = 3). Addition of serosal DNDS (3 mM) and acetazolamide (100 μM) reduced $I_{sc}$ to 6.3 ± 0.3 and 5.1 ± 0.2 μA/cm², respectively, suggesting that 1-EBIO stimulates HCO3− secretion across wt HBE.

Effect of Mucosal UTP on Cl− Secretion Across HBE

As shown in Fig. 7, mucosal UTP induced a transient increase in $I_{sc}$ followed by a sustained plateau at a reduced current level similar to what has been previously described (37). In 44 experiments, subsequent to amiloride, mucosal UTP (100 μM) increased $I_{sc}$ from 12.8 ± 0.7 to 28.2 ± 1.3 μA/cm² which was then followed by a sustained plateau level at 16.6 ± 0.9 μA/cm². Addition of serosal DNDS (3 mM)- and acetazolamide (100 μM) reduced $I_{sc}$ to 19.3 ± 0.9 and 9.9 ± 0.5 μA/cm², respectively. These results demonstrate that pharmacological activation of basolateral membrane K+ channels is an alternative strategy to stimulate Cl− secretion across human bronchial epithelia.

*Fig. 6. Effect of 1-EBIO (1 mM) and forskolin (10 μM) on $I_{sc}$ in wt CFTR-expressing HBE. A: subsequent to amiloride (10 μM), forskolin induced a sustained increase in $I_{sc}$ that was further potentiated by the addition of 1-EBIO. Addition of CTX (50 nM, serosal) and bumetanide (20 μM) inhibited the forskolin and 1-EBIO-induced $I_{sc}$. B: subsequent to amiloride, 1-EBIO induced a sustained increase in $I_{sc}$ that was further increased by the addition of forskolin. Addition of CTX (50 nM, serosal) and bumetanide (20 μM) inhibited the forskolin and 1-EBIO-induced $I_{sc}$. Dashed lines, zero current level.*
μA/cm². Subsequent addition of bumetanide reduced I_{sc} to 9.4 ± 0.7 μA/cm². In the absence of mucosal and serosal Cl⁻, mucosal UTP increased I_{sc} an average of 4.5 ± 0.6 μA/cm², suggesting that UTP induces a small amount of HCO₃⁻ secretion across wt HBE. Consistent with this notion, in the absence of both Cl⁻ and HCO₃⁻, mucosal UTP increased I_{sc} by only 0.5 ± 0.2 μA/cm². Similarly, Smith and Welsh (47) demonstrated that the Ca²⁺ ionophore A-23187 increased I_{sc} in the absence of Cl⁻, across wt HBE.

In the T84 colonic cell line the response to the Ca²⁺-mediated agonist, carbachol, is potentiated by both Cl⁻ channel activators, including forskolin (17), NS004 and 1-EBIO (13), and psoralens (14), as well as compounds that impinge on second messenger pathways such as wortmannin (phosphatidylinositol 3-kinase) (52) and arachidonyl trifluoromethyl ketone (AACOCF₃) [phospholipase A2 (PLA₂)] (2, 11). Indeed, we demonstrated that the Ca²⁺ ionophore A-23187 increased I_{sc} across T84 cells but also induced a sustained phase to the carbachol response. Potentiation of either the transient or sustained phase of a Ca²⁺-mediated response in HBE would be expected to be of therapeutic benefit. Therefore, we evaluated the effect of these compounds on the I_{sc} response to mucosal UTP across wt HBE. In contrast to previous results from T84 cells, the effect of UTP on Cl⁻ secretion across wt HBE was unchanged in the presence of NS004 (10 μM; ΔI_{sc} = 10.9 ± 2.7 μA/cm²; n = 4), 1-EBIO (1 mM; ΔI_{sc} = 5.0 ± 0.2 μA/cm²; n = 9), AACOCF₃ (100 μM; ΔI_{sc} = 8.9 ± 1.6 μA/cm²; n = 6) or wortmannin (100 nM, ΔI_{sc} = 10.1 ± 2.3 μA/cm²; n = 7). These results suggest that the pharmacological potentiation of Ca²⁺-mediated agonists observed in other model Cl⁻ secretory systems cannot be readily extrapolated to human bronchial systems.

The response to mucosal UTP (100 μM) in the presence of forskolin is shown for one experiment in Fig. 8A. Subsequent to forskolin, addition of mucosal UTP induced a further increase in I_{sc} that was followed by a decline to below the initial forskolin plateau level. In a total of 30 monolayers, mucosal UTP increased I_{sc} by only 7.0 ± 1.2 μA/cm², with the plateau current level being 4.9 ± 0.6 μA/cm² below the sustained forskolin-induced current level. These results demonstrate that the response to mucosal UTP is not potentiated by forskolin. Indeed, the mucosal UTP-induced increase in I_{sc} in the presence of forskolin is smaller than that observed in the absence of forskolin (P < 0.001). Also, the net effect of UTP is a decrease in total outward current. The net decrease in outward current observed could be due to an inhibition of Cl⁻ secretion or a stimulation of K⁺ secretion. Indeed, we have previously shown that mucosal UTP inhibits the Ba²⁺- and quinine-sensitive basolateral G_{sc} likely responsible for maintaining the driving force for Cl⁻ secretion in the presence of forskolin (12) (Fig. 2). To determine whether the decrease in I_{sc} observed was specific for UTP, we determined the effect of the Ca²⁺-ATPase inhibitor, thapsigargin. Similar to mucosal UTP, following establishment of a sustained forskolin response, thapsigargin (1 μM) induced an initial small

Fig. 7. Effect of mucosal UTP on I_{sc} across wt HBE. Subsequent to amiloride (10 μM), mucosal UTP (100 μM) induced a transient increase in I_{sc} followed by a sustained plateau that was sensitive to inhibition by bumetanide (20 μM). Dashed line, zero current level.

Fig. 8. Effect of mucosal UTP on basolateral K⁺ conductance. A: subsequent to amiloride (Amil); 10 μM, forskolin (Forsk) induced a sustained increase in I_{sc}. Addition of mucosal UTP (100 μM) induced a further transient increase in I_{sc} followed by a decline to below the forskolin-induced I_{sc} level. B: addition of CTX (50 nM, serosal) had no effect on the forskolin-dependent increase in I_{sc}. Although it completely inhibited the UTP-dependent increase in I_{sc}. CTX had no effect on the UTP-dependent decrease in I_{sc}. In both cases, bumetanide (20 μM) inhibited the forskolin-dependent increase in I_{sc}. Dashed lines, zero current level.
increase in $I_{sc}$ ($\Delta I_{sc} = 3.9 \pm 0.8 \mu A/cm^2; n = 10$) followed by a decline in current to $4.3 \pm 0.9 \mu A/cm^2$ below the sustained forskolin current level. This result indicates the decrease in current is observed is independent of receptor-mediated effects.

We next determined whether the initial increase in $I_{sc}$ induced by UTP is due to the activation of a CTX-sensitive basolateral membrane K$^+$ channel. As shown in Fig. 5B, CTX (50 nM) completely inhibited the initial increase in $I_{sc}$ induced by mucosal UTP, whereas the apparent inhibitory phase was unaffected. In seven monolayers, the initial increase induced by UTP in the presence of CTX averaged $0.4 \pm 0.4 \mu A/cm^2$, whereas in seven paired experiments, carried out on the same day on monolayers from the same patient, UTP induced a significantly greater increase in $I_{sc}$, averaging $12.9 \pm 3.9 \mu A/cm^2 (P < 0.001)$. These results demonstrate that the initial increase in Cl$^-$ secretion induced by UTP is due to the activation of a basolateral membrane, CTX-sensitive K$^+$ channel. Prior microelectrode studies confirm that mucosal ATP induces a large decrease in basolateral membrane resistance with no change in the electromotive force of the basolateral membrane, consistent with activation of both basolateral $G_K$ and $G_{Cl}$ (6). In this case, inhibition of $G_K$ with CTX would result in a depolarization of the basolateral membrane toward the Cl$^-$ reversal potential ($E_{Cl}$) such that $I_{sc}$ is decreased.

Ca$^{2+}$-Dependent K$^+$ Secretion Across wt and ΔF-508 HBE

Our results suggest that mucosal UTP may be stimulating K$^+$ secretion across HBE. Indeed, Clarke et al. (7) have shown that luminal ATP is capable of stimulating K$^+$ secretion across human airway; demonstrating that the driving force across the apical membrane favors K$^+$ secretion. To evaluate whether we were observing a similar phenomenon, we established a serosa-to-mucosa K$^+$ gradient (145–5 mM) across the epithelium while inhibiting Na$^+$ absorption with amiloride (10 μM). As shown in Fig. 9A, addition of mucosal UTP (100 μM) induced a large, transient increase in inward $I_{sc}$ consistent with stimulation of K$^+$ secretion. Sequential addition of the K$^+$ channel blockers glibenclamide (100 μM) and Ba$^{2+}$ (5 mM) to the mucosal solution resulted in an inhibition of the remaining $I_{sc}$ as well as the basal $I_{sc}$. In contrast, addition of forskolin had no effect on K$^+$ secretion across HBE (n = 3, data not shown). In four experiments, UTP induced a peak increase in $I_{sc}$ of $197 \pm 21 \mu A/cm^2$. Due to the transient nature of these responses, further experiments were carried out using the Ca$^{2+}$-ATPase inhibitor thapsigargin as a means of raising intracellular Ca$^{2+}$. However, even in the presence of thapsigargin, the K$^+$ secretory currents tended to be somewhat transient in nature (see Fig. 9B). In 12 experiments, thapsigargin (1 μM) increased $I_{sc}$ from $33 \pm 4$ to $142 \pm 14 \mu A/cm^2$ followed by a decline to $77 \pm 8 \mu A/cm^2$. The combination of mucosal glibenclamide and Ba$^{2+}$ reduced this $I_{sc}$ to $21 \pm 2 \mu A/cm^2$ (n = 9). In the additional three experiments, the effect of mucosal CTX addition was evaluated. CTX (50 nM) reduced $I_{sc}$ from $72 \pm 2$ to $32 \pm 11 \mu A/cm^2$. As Ba$^{2+}$ and CTX will not cross the epithelium, these results demonstrate the existence of a K$^+$ conductance in the apical membrane of HBE. In contrast, the cAMP-dependent K$^+$ channel blocker, 293B (100 μM), had no effect on the Ca$^{2+}$-dependent K$^+$ secretion (n = 3, data not shown), con-

Fig. 9. Effect of mucosal UTP (100 μM, A) and thapsigargin (1 μM, B) on K$^+$ secretion across wt CFTR (A)- and ΔF508 CFTR (B)-expressing HBE. After establishment of a serosa-to-mucosa K$^+$ gradient across the HBE monolayer in the absence of Cl$^-$ (120 mM potassium gluconate,5 mM potassium gluconate) and inhibition of Na$^+$ absorption with amiloride (10 μM), both mucosal UTP (A) and thapsigargin (B) induced an inward K$^+$ secretory current. Addition of glibenclamide (Giben; 100 μM) to the mucosal solution inhibited this K$^+$ current in both wt CFTR (A) and ΔF508 CFTR (B)-expressing HBE. The subsequent addition of Ba$^{2+}$ (5 mM) to the mucosal solution resulted in a further decrease in K$^+$ current. Dashed lines, zero current level.
sistent with the lack of effect of forskolin in these experiments.

The observation that glibenclamide inhibited the Ca\(^{2+}\)-dependent K\(^+\) secretory response led us to determine whether increased Ca\(^{2+}\) would similarly stimulate K\(^+\) secretion across ΔF508 HBE in a glibenclamide-dependent fashion. As shown in Fig. 9B, thapsigargin stimulated K\(^+\) secretion across ΔF508 HBE that was sensitive to block by mucosal glibenclamide (100 μM). The subsequent addition of mucosal Ba\(^{2+}\) (5 mM) further inhibited I\(_{sc}\). In six experiments, thapsigargin increased I\(_{sc}\) from 56 ± 12 to 235 ± 28 μA/cm\(^2\). Addition of glibenclamide and Ba\(^{2+}\) reduced I\(_{sc}\) to 56 ± 6 and 18 ± 1 μA/cm\(^2\), respectively. These results demonstrate that Ca\(^{2+}\)-dependent agonists similarly activate an apical membrane, glibenclamide-sensitive G\(_k\) in ΔF508 HBE. Also, these results suggest that the magnitude of the UTP-dependent Cl\(^-\) secretory response observed may be underestimated, as any simultaneous UTP-dependent K\(^+\) secretory response would produce a current of opposite polarity.

Effect of Pharmacological Modulators on Cl\(^-\) Secretion Across ΔF-HBE

The above data demonstrate that the pharmacological agents NS004, genistein, 8-MOP, and 1-EBIO stimulate sustained transepithelial Cl\(^-\) secretory responses across wt CFTR-expressing HBE. We next determined whether these agonists would similarly modulate Cl\(^-\) secretion across ΔF508 CFTR-expressing HBE (ΔF-HBE). We initially determined the effect of forskolin and mucosal UTP on I\(_{sc}\) across ΔF-HBE. Consistent with the CF phenotype, forskolin increased I\(_{sc}\) by only 1.2 ± 0.3 μA/cm\(^2\) (n = 30). Mucosal UTP also induced a smaller I\(_{sc}\) response compared with wt CFTR-expressing HBE, with the peak response averaging 5.8 ± 0.7 μA/cm\(^2\) (n = 13) followed by a sustained plateau 2.0 ± 0.4 μA/cm\(^2\) above baseline. In contrast to the results reported here, others have reported similar or enhanced responses to Ca\(^{2+}\)-mediated agonists across ΔF508-expressing airway (6, 32, 33, 37).

1-EBIO. Before evaluating the effects of 1-EBIO on transepithelial Cl\(^-\) secretion across ΔF-HBE, we determined whether the basolateral membrane K\(^+\) conductance in ΔF-HBE would respond to pharmacological (1-EBIO) modulation. The mucosal membrane was permeabilized with nystatin and a serosa-to-mucosa K\(^+\) gradient established across the epithelium to measure serosal membrane K\(^+\) currents (I\(_K\); see METHODS). After nystatin permeabilization, 1-EBIO (1 mM) increased I\(_K\) from 11.3 ± 1.2 to 31.0 ± 2.8 μA/cm\(^2\) (n = 8), and this was inhibited by CTX (50 nM; 13.8 ± 1.2 μA/cm\(^2\)), consistent with activation of a basolateral membrane Ca\(^{2+}\)-dependent K\(^+\) conductance (10, 15). This response to 1-EBIO was similar in magnitude to that induced by mucosal UTP (ΔI\(_K\) = 31.2 ± 6.9 μA/cm\(^2\), n = 9).

We next determined the effect of 1-EBIO (1 mM) on transepithelial Cl\(^-\) secretion, subsequent to amiloride, in ΔF-HBE. Despite the fact that 1-EBIO activates a basolateral K\(^+\) conductance in ΔF-HBE, it had no effect on I\(_{sc}\). In 15 experiments the ΔI\(_{sc}\) averaged only 0.1 ± 0.1 μA/cm\(^2\). These results demonstrate that activation of the basolateral membrane K\(_{Ca}\) is insufficient to modulate Cl\(^-\) secretion across ΔF-HBE.

8-MOP. We next evaluated the effect of 8-MOP on ion transport across ΔF-HBE, subsequent to inhibition of Na\(^+\) absorption with amiloride. Similar to 1-EBIO, 8-MOP failed to significantly increase I\(_{sc}\), subsequent to amiloride. In four monolayers, amiloride reduced I\(_{sc}\) from 44.1 ± 5.1 to 1.8 ± 0.2 μA/cm\(^2\), with the subsequent addition of 8-MOP (10 μM) increasing I\(_{sc}\) to only 2.2 ± 0.4 μA/cm\(^2\).

NS004. Because NS004 stimulates Cl\(^-\) secretion across HBE-expressing wt CFTR (Fig. 4), we determined whether this proposed CFTR activator would stimulate Cl\(^-\) secretion in ΔF-HBE. As shown in Fig. 10A, subsequent to amiloride, NS004 (10 μM) had little effect on I\(_{sc}\). The subsequent addition of forskolin (10 μM) induced an increase in I\(_{sc}\), which was sensitive to bumetanide. In 13 experiments NS004 failed to significantly increase I\(_{sc}\) (0.4 ± 0.3 μA/cm\(^2\)), whereas the subsequent addition of forskolin increased I\(_{sc}\) (2.3 ± 0.3 μA/cm\(^2\)). The effect of NS004 on I\(_{sc}\) was also evaluated subsequent to forskolin addition (Fig. 10B). Forskolin increased I\(_{sc}\) an average of 1.4 ± 0.3 μA/cm\(^2\). In contrast to NS004 alone, addition of NS004 subsequent to forskolin induced a significantly greater, bumetanide-sensitive increase, in I\(_{sc}\) (2.1 ± 0.3 μA/cm\(^2\), n = 21, P < 0.001). These results suggest that forskolin and NS004 act in a synergistic fashion to stimulate Cl\(^-\) secretion across ΔF-HBE (Fig. 10C).

Genistein. The effect of genistein (50 μM) on Cl\(^-\) secretion across ΔF-HBE is shown in Fig. 11. Subsequent to amiloride, genistein induced a small, bumetanide-sensitive increase in I\(_{sc}\). In contrast to our results with NS004, addition of forskolin caused no further increase in I\(_{sc}\) following genistein. In 11 experiments, genistein increased I\(_{sc}\) an average of 2.5 ± 0.5 μA/cm\(^2\) (P < 0.01), with the subsequent addition of forskolin increasing I\(_{sc}\) by only an additional 0.4 ± 0.5 μA/cm\(^2\). In an additional nine experiments carried out on monolayers from the same culture, the order of forskolin and genistein addition were reversed. In these monolayers forskolin increased I\(_{sc}\) by 0.5 ± 0.2 μA/cm\(^2\), with genistein further increasing I\(_{sc}\) by only 0.7 ± 0.5 μA/cm\(^2\).

Effect of Pharmacological Activators on ΔF-HBE Cultured at 26°C

Denning et al. (9) demonstrated that ΔF508 CFTR was a temperature-sensitive mutant, i.e., ΔF508 CFTR could escape the degradative pathway and be expressed at the plasma membrane if cells expressing this mutation were grown at a reduced temperature (26°C). Thus we determined whether 1-EBIO, NS004, or genistein would stimulate a Cl\(^-\) secretory response in ΔF-HBE after incubating the monolayers at 26°C for 24 h. The results of these experiments are shown in Fig. 12. Although 1-EBIO (1 mM) had no effect on ΔF-HBE grown at 37°C, it stimulated a small, sus-
tained increase in $I_{sc}$ following incubation at 26°C (Fig. 12A). In four experiments this $I_{sc}$ response averaged $1.5 \pm 0.2 \mu A/cm^2$, which is significantly greater than when the cells were grown at 37°C ($P < 0.01$). Similar to these results, both NS004 (10 μM, fig. 12B) and genistein (50 μM, Fig. 12C) caused significantly greater $I_{sc}$ responses following incubation at 26°C ($2.0 \pm 0.3 \mu A/cm^2$, $n = 7$, $P < 0.01$; and $8.3 \pm 2.4 \mu A/cm^2$, $n = 6$, $P < 0.01$, respectively). The effect of forskolin on $I_{sc}$, subsequent to NS004 and genistein, was also evaluated in these experiments (Fig. 12, B and C). Subsequent to NS004, forskolin induced a further increase in $I_{sc}$ of $1.8 \pm 0.2 \mu A/cm^2$ ($n = 7$), consistent with our results when the cells were grown at 37°C. However, following genistein stimulation, forskolin caused a small decrease of $1.4 \pm 0.5 \mu A/cm^2$ ($n = 6$) in $I_{sc}$. Finally, forskolin alone caused a small increase in $I_{sc}$ of $2.1 \pm 0.9 \mu A/cm^2$ ($n = 8$), although this was not significantly greater than the response observed following culture at 37°C.

Effect of 1-EBIO and Forskolin on ΔF508/2789 +5G→A HBE

Highsmith et al. (24) identified a splice site mutation in exon 14b of CFTR (2789 +5G→A), which results in a frameshift of CFTR mRNA predicted to result in early termination of the CFTR protein. In patients homozygous for this mutation, ~4% of normal CFTR mRNA was produced and was associated with mild
disease (24). We evaluated the effect of 1-EBIO and forskolin on a total of five HBE monolayers from a patient heterozygous for this mutation, the other allele being \( \text{DF508} \). In contrast to our results on homozygous \( \text{DF508} \) monolayers, 1-EBIO (1 mM) induced a sustained increase in \( I_{sc} \) of 2.1 ± 0.3 \( \mu \)A/cm\(^2\) (\( n = 3 \), \( P < 0.05 \)). In two additional monolayers, forskolin (10 \( \mu \)M) increased \( I_{sc} \) by 4.5 \( \mu \)A/cm\(^2\), and this was further increased 2.1 \( \mu \)A/cm\(^2\) by the addition of 1-EBIO (1 mM). These results suggest that as little as 2% of wt CFTR mRNA generates sufficient protein to be pharmacologically manipulated.

Effect of NS004, 8-MOP, 1-EBIO, and Genistein on \( \text{Na}^+ \) Absorption Across \( \text{DF508} \)

Both a diminished \( \text{Cl}^- \) secretory response to cAMP-mediated agonists as well as hyperabsorption of \( \text{Na}^+ \) characterize CF. Thus it is critical to evaluate the effects of proposed pharmacological modulators of \( \text{Cl}^- \) secretion on \( \text{Na}^+ \) transport as well. We evaluated the effects of NS004, 8-MOP, 1-EBIO, and genistein on \( \text{Na}^+ \) transport across \( \text{DF508} \). We used \( \text{DF508} \) for these studies to avoid the confounding effects these compounds have on both \( \text{Cl}^- \) and \( \text{HCO}_3^- \) secretion. As detailed above, these pharmacological agents have very little effect on \( \text{Cl}^- \) secretion across \( \text{DF508} \). As shown in Fig. 13, NS004 and 8-MOP (10 \( \mu \)M) had no effect on \( \text{Na}^+ \) absorption across \( \text{DF508} \) (\( I_{sc} = -0.7 \pm 0.7 \) \( \mu \)A/cm\(^2\); 1.2 ± 0.5 \( \mu \)A/cm\(^2\), respectively, \( n = 5 \) for both). In contrast, both 1-EBIO (1 mM, Fig. 13C) and genistein (50 \( \mu \)M, Fig. 13D) increased \( I_{sc} \) by 3.6 ± 0.6 \( \mu \)A/cm\(^2\) (\( n = 8 \), \( P < 0.001 \)) and 10.1 ± 1.3 \( \mu \)A/cm\(^2\) (\( n = 11 \), \( P < 0.001 \)), respectively. These results are summarized in Fig. 13E. Subsequent addition of amiloride reduced \( I_{sc} \) to 4.7 ± 1.0 and 4.1 ± 0.7 \( \mu \)A/cm\(^2\) in the presence of 1-EBIO and genistein, respectively. These values are not different from the amiloride-sensitive baseline seen in the absence of agonist (5.3 ± 0.2 \( \mu \)A/cm\(^2\), \( n = 112 \)). These results are consistent with 1-EBIO and genistein stimulating \( \text{Na}^+ \) absorption across \( \text{DF508} \).

DISCUSSION

When comparing the effects of pharmacological modulators of transepithelial ion transport between wt and \( \text{DF508} \) CFTR-expressing human airway, it is important to consider the source of the material. Although all of our wt CFTR-expressing HBE were from transplant tissue, only one was from a bronchiectasis patient chronically colonized with \( \text{Pseudomonas aeruginosa} \), similar to our CF cultures. Thus it could be argued that the milieu of the wt CFTR- vs. \( \text{DF508} \) CFTR-expressing...
HBE, before culture, may impact the subsequent results. This is unlikely, however, because our monolayers were studied 3–5 wk following acquisition of the bronchial material, having been cultured in two separate growth media. A second consideration is that the cells cultured from these two patient populations are distinct and therefore respond differently. However, both our wt CFTR and ΔF508 CFTR-expressing HBE were Na⁺ absorptive in the basal state, consistent with a surface cell phenotype. In addition, our CF cultures displayed both the hallmark hyperabsorption of Na⁺ and diminished Cl⁻ secretory responses associated

Fig. 13. Effect of NS004 (10 μM, A), 8-methoxypsoralen (8-MOP; 10 μM, B), 1-EBIO (1 mM, C), and genistein (50 μM, D) on basal Na⁺ absorption in ΔF508 CFTR-expressing HBE. E: average changes in Na⁺ current (ΔI_{Na⁺}). Whereas NS004 had no effect on Na⁺ absorption, both 1-EBIO and genistein induced significant increases in Na⁺ absorption (*P < 0.05).
with in vivo measurements from CF patients (32, 33). Recently, both Engelhardt and colleagues (20, 61) and Dupuit et al. (18) have shown that, after reconstitution (in xenografts) of epithelial cells derived from diseased nasal or bronchial epithelium, the distribution of airway cell phenotypes is not different than normal airway. This suggests that a progenitor cell population is selected with in vitro culture and that expansion and differentiation of this population normalizes the morphological differences typically present in the diseased lung. Finally, we have performed scanning electron microscopy (SEM) and immunostaining for markers of cell differentiation in airway cultures derived from both CF and non-CF human airway. By SEM, the majority of cells in all filters examined had features of differentiated airway cells, namely abundant microvilli and cilia, and apical expression of the membrane mucin MUC1 (data not shown). Collectively, these data suggest that the differences in ion transport between the CF and non-CF airway cell filters do not reflect the inflammatory milieu of the underlying disease or differences in epithelial cell phenotype but rather are associated with the CF genotype.

Pharmacological Modulation of Cl\textsuperscript{−} Secretion

We previously demonstrated that the proposed CFTR openers, NS004 and 8-MOP, failed to modulate Cl\textsuperscript{−} secretion across T84 cell monolayers (13, 14). In contrast, the dual K\textsubscript{Ca} (hIK1) and CFTR opener, 1-EBIO, induced a sustained Cl\textsuperscript{−} secretory response in T84 cells (13, 15). Nguyen et al. (41) first demonstrated that flavones were capable of stimulating Cl\textsuperscript{−} secretion across T84 monolayers. More recently, it has been shown that the related compound, genistein, also stimulates Cl\textsuperscript{−} secretion (28), perhaps via a direct effect on CFTR (25, 54). However, these compounds have not been investigated on primary cultures of human airway epithelia and in particular those expressing the ΔF508 CFTR mutation. We demonstrate that NS004, 8-MOP, genistein, and 1-EBIO all stimulate Cl\textsuperscript{−} secretion across wt CFTR-expressing HBE. We also demonstrate that NS004 and genistein can induce a small Cl\textsuperscript{−} secretory response in ΔF508 CFTR-expressing HBE (ΔF-HBE). The diminished response in ΔF-HBE leads us to conclude that these agonists are activating CFTR. Importantly, these results further suggest that pharmacological agents can directly modulate CFTR expressed at levels found in primary cultures of airway epithelia. An alternative possibility (which we do not favor) is that all these agents activate a distinct Cl\textsuperscript{−} channel that requires wt CFTR for both normal function and pharmacological regulation. Although genistein alone stimulated Cl\textsuperscript{−} secretion across ΔF-HBE, the effect of NS004 required the addition of forskolin. These results indicate that NS004 and genistein modulate Cl\textsuperscript{−} secretion via different mechanisms. Thus we demonstrate, for the first time, that pharmacological modulators of CFTR can stimulate Cl\textsuperscript{−} secretion across human airway homozygous for the ΔF508 CF mutation. This is consistent with the hypothesis that ΔF508 CFTR is capable of escaping the degradative pathway and being expressed at the apical membrane in human bronchial epithelium. Although we have not quantified the level of ΔF508 CFTR expression in our cells, previous reports have demonstrated that ΔF508 CFTR is expressed at the apical membrane of airway (19, 31). Indeed, Kalin et al. (31) recently demonstrated that the expression and localization of ΔF508 CFTR could not be distinguished from wt CFTR in intestinal and respiratory epithelia. Thus the decreased current response observed in our cultures may in part reflect the decreased open probability (P\textsubscript{o}) associated with ΔF508 CFTR (44).

It has been proposed that pharmacological intervention designed to increase the trafficking of ΔF508 CFTR to the apical membrane would be therapeutically beneficial. One means of testing this strategy is to culture ΔF508 CFTR-expressing cells at 26°C (9). We demonstrate that after incubation of ΔF-HBE at 26°C for 24 h, the Cl\textsuperscript{−} secretory response to NS004, genistein, and 1-EBIO are all potentiated. These results further demonstrate that pharmacological agents are capable of activating ΔF508 CFTR in human airway epithelium.

An important question is, How much CFTR must be expressed at the apical membrane to pharmacologically elevate anion secretion? Although we have not quantified the levels of CFTR in the apical membrane of our cultures, our studies on the CFTR splice site mutation 2789 +5G→A shed some light on this question. Highsmith et al. (24) demonstrated that airway cells homozygous for the splice site mutation 2789 +5G→A express ~4% wt CFTR mRNA, whereas the remainder of the CFTR is stopped prematurely during translation. In cells heterozygous for the 2789 +5G→A mutation (the other allele being ΔF508), 1-EBIO induced a sustained Cl\textsuperscript{−} secretory response, whereas in homozygous ΔF508 airway cells 1-EBIO had no measurable effect. Similarly, subsequent to forskolin, 1-EBIO further increased I\textsubscript{cl}. These results suggest that as little as 2% of wt CFTR mRNA is required to generate sufficient protein to be pharmacologically manipulated.

CFTR is Rate Limiting in Both wt and ΔF508 HBE

We previously reported that NS004 and 8-MOP failed to stimulate transepithelial Cl\textsuperscript{−} secretion across both the model Cl\textsuperscript{−} secretory epithelia, T84, and primary cultures of MTE, despite the fact that both NS004 and 8-MOP increased apical membrane Cl\textsuperscript{−} conductance (G\textsubscript{Cl}) in nystatin permeabilized monolayers (13, 14). Recent impedance analysis data support the conclusion that NS004 and 8-MOP increase apical membrane conductance in T84 cells (Bridges RJ, unpublished observations). Prior activation of the basolateral membrane K\textsubscript{Ca} channel by either 1-EBIO or carbachol is required for a sustained Cl\textsuperscript{−} secretory response to NS004 (13) and 8-MOP (14). These results led us to conclude that the basolateral membrane was rate limiting to Cl\textsuperscript{−} secretion across T84 cells. In con-
rst to these results, we demonstrate that NS004 stimulates a sustained Cl\textsuperscript{−} secretory response across HBE (Fig. 4), indicative of CFTR being rate limiting for Cl\textsuperscript{−} secretion. We further demonstrate that this Cl\textsuperscript{−} secretory response is not due to activation of BK channels in the basolateral membrane by NS004. Our conclusion is further supported by the observation that inhibition of G\textsubscript{K} results in a diminished response to NS004. In this instance we have shifted the rate-limiting conductance by artificially decreasing basolateral membrane K\textsuperscript{+} conductance. Our hypothesis is further strengthened by the observation that an additional activator of apical G\textsubscript{C0}, 8-MOP, similarly stimulates Cl\textsuperscript{−} secretion across HBE. The Cl\textsuperscript{−} secretory response observed cannot be wholly explained by the hyperpolarization of the apical membrane by amiloride, as NS004 failed to induce Cl\textsuperscript{−} secretion across MTE in the presence of amiloride (13). Also, we demonstrate that NS004 and 8-MOP, two compounds that have no effect on Na\textsuperscript{+} transport across HBE (Fig. 13), induce a small but significant increase in I\textsubscript{sc} in the absence of amiloride. Finally, CFTR modulators had little effect on Cl\textsuperscript{−} secretion across Δ\textit{V}-HBE, consistent with CFTR being rate limiting.

Our results suggest that a basolateral membrane G\textsubscript{K} is constitutively active, thereby providing the driving force necessary for Cl\textsuperscript{−} secretion in response to the CFTR openers, NS004 and 8-MOP. We speculate this conductance is responsible for maintaining the driving force for Na\textsuperscript{+} entry across the apical membrane in the nonstimulated state. Addition of amiloride has been shown to stimulate Cl\textsuperscript{−} secretion across human airway epithelia, due to a hyperpolarization of both apical (V\textsubscript{a}) and basolateral membranes with a concomitant increase in apical membrane fractional resistance (58). These data indicate that the basolateral membrane is not downregulated in parallel with the apical Na\textsuperscript{+} conductance. Thus this basolateral G\textsubscript{K} maintains the electrochemical driving force for Cl\textsuperscript{−} exit in the presence of amiloride. These data also highlight the critical need to evaluate potential CFTR openers in the appropriate context, since, in the absence of a full complement of apical and basolateral conductances, the electrochemical driving force acting on Cl\textsuperscript{−} may be inappropriate for identifying Cl\textsuperscript{−} channel modulators.

**HCO\textsubscript{3}\textsuperscript{−} Secretion Across Human Airway**

Smith and Welsh (47) previously demonstrated that forskolin stimulates an increase in I\textsubscript{sc} across human airway epithelia in the absence of mucosal and serosal Cl\textsuperscript{−}. In further studies on canine airway, these authors (47) demonstrated that this increase in I\textsubscript{sc} depended on both the generation of HCO\textsubscript{3}\textsuperscript{−} via carbonic anhydrase and the transport of H\textsuperscript{+} across the basolateral membrane via a Na\textsuperscript{+}/H\textsuperscript{+} exchanger (amiloride sensitivity). More recently we demonstrated that the human airway serous cell line, Calu-3, secretes HCO\textsubscript{3} in response to forskolin (16). Whereas a small component of this HCO\textsubscript{3} transport was dependent on carbonic anhydrase, the majority of transport was inhibited by serosal DNDS (16). DNDS has recently been shown to inhibit the electrogenic transport of HCO\textsubscript{3} via a Na\textsuperscript{+}/HCO\textsubscript{3} co-transporter (NBC) (4, 56). Thus we posited that HCO\textsubscript{3} entered via the NBC and exited across the apical membrane via CFTR (16). Here, we confirm the earlier work of Smith and Welsh (47) by demonstrating that forskolin stimulates an acetazolamide- and EIPA-sensitive increase in I\textsubscript{sc} in the absence of Cl\textsuperscript{−}, suggesting this is likely a HCO\textsubscript{3} secretory response (Fig. 1). In addition, we demonstrate that DNDS inhibits this Cl\textsuperscript{−}-independent I\textsubscript{sc} response, consistent with a serosal NBC playing a critical role in transepithelial HCO\textsubscript{3} secretion. RT-PCR and sequence data, as well as RNase protection assays, have demonstrated the expression of an NBC in HBE (Gangopadhyay NN and Bridges RJ, unpublished observations). The present data support our proposal that HCO\textsubscript{3} exits across the apical membrane via CFTR, as known pharmacological modulators of CFTR, NS004 and genistein, also stimulate HCO\textsubscript{3} secretion across HBE in a Cl\textsuperscript{−}-independent manner. We conclude that these modulators have little if any effect on Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} channels based on their modest Cl\textsuperscript{−} secretory capacity in Δ\textit{V}-HBE. In addition, HCO\textsubscript{3} secretion occurs in the absence of luminal Cl\textsuperscript{−}, which argues against a role for an apical Cl\textsuperscript{−}/HCO\textsubscript{3} exchanger. Thus the simplest interpretation of these results is that HCO\textsubscript{3} secretion occurs via CFTR. We therefore propose a model that confirms and extends the one originally proposed by Smith and Welsh (47) for HBE by including an NBC in the serosal membrane that facilitates HCO\textsubscript{3} entry and by providing additional pharmacological data supporting the role of CFTR as the HCO\textsubscript{3} conductive pathway in the apical membrane.

**K\textsuperscript{+} Channels Involved in Ion Transport Across HBE**

As is well known, the maintenance of both Na\textsuperscript{+} absorption and Cl\textsuperscript{−} secretion requires the coordinate regulation of an apical membrane conductance (ENaC, CFTR) coupled with one or more basolateral membrane K\textsuperscript{+} conductances. Indeed, we demonstrate that modulation of these K\textsuperscript{+} conductances can stimulate both Na\textsuperscript{+} absorption and Cl\textsuperscript{−} secretion, supporting our proposal that G\textsubscript{K} can serve as an alternate therapeutic target in airway disease. Unfortunately, these K\textsuperscript{+} conductances have been little studied in human airway. We demonstrate that mucosal UTP activates a CTX-sensitive K\textsuperscript{+} conductance in the basolateral membrane. Thus this K\textsuperscript{+} channel is already the target of therapeutic intervention, since UTP is currently being evaluated in clinical CF trials (32, 33). Importantly, this channel is also one of the targets of 1-EBIO. This channel likely corresponds to the recently cloned hIK1 (29, 30), since we have recently demonstrated, by Northern blot analysis, that hIK1 is expressed in both Calu-3 cells (21) and HBE (unpublished observations) and that this channel is directly activated by 1-EBIO (50).

Similar to our previous findings on T84 cells (15), the forskolin-stimulated Cl\textsuperscript{−} secretory response was par-
tially inhibited by the cAMP-dependent K+ channel blocker, 293B (Fig. 2) (34). Although it was originally proposed that 293B inhibited the min-K channel (I_{KCl}) (49), more recent evidence suggests that the molecular target of 293B is actually K_LQT1 (35). Indeed, K_LQT1 has been shown to be expressed in both T84 cells and HBE (8) (unpublished observations), consistent with our blocker profile. In contrast to these results, MacVinish et al. (36) reported that the cAMP response in murine nasal epithelia was insensitive to 293B. However, we have observed a 293B-inhibitable current in response to forskolin in primary cultures of MTE (Devor DC, unpublished observations). These results suggest that murine nasal epithelia may not be an adequate model for human bronchial epithelia with regard to K+ channel expression. Also, we observed no effect of NS004 on Cl− secretion across MTE (13), further suggesting that murine airway is an inadequate model for predicting human airway ion transport. Indeed, the observation that murine airway epithelia are unaffected in CFTR knockout mice supports this supposition.

In addition to basolateral membrane K+ conductances, we demonstrate a significant K+ conductance in the apical membrane as well. This conductance was activated by increasing cellular Ca2+ but not cAMP, similar to what has recently been reported by Clarke et al. (7). Whereas our results do not distinguish between secretion and absorption, Clarke et al. (7) have demonstrated that the electrochemical driving force acting on K+ favors secretion across the apical membrane. Thus, whereas CFTR has been proposed to interact with ROMK at the apical membrane of kidney epithelia, thereby conferring glibenclamide sensitivity (40), we demonstrate that apical membrane K+ conductance expression and blocker pharmacology are independent of wt CFTR expression (Fig. 9).

**Pharmacological Modulation of Na+ Absorption**

CF is characterized not only by a diminished Cl− secretory response to cAMP-mediated agonists, but also by a hyperabsorption of Na+. Our in vitro airway cell system recapitulates this Na+ hyperabsorption. It has been proposed that this increased Na+ absorption may contribute to the dehydration of airway secretions and impairment of mucociliary clearance (38). This has led to clinical trials designed to determine whether pharmacological inhibition of Na+ transport would be therapeutically beneficial in CF patients (22, 51). Ideally then, any proposed modulator of CFTR would have either no effect on Na+ transport or would simultaneously inhibit Na+ absorption, thus creating a favorable driving force for Cl− secretion. We recently demonstrated that the Cl− secretagogue UTP inhibits Na+ absorption (12), suggesting this agonist could be utilized in the absence of amiloride. Here we demonstrate that the CFTR openers, NS004 and 8-MOP, have no effect on Na+ transport across ΔF-HBE (Fig. 13). In contrast, the K_{Ca} opener, 1-EBIO, increases Na+ absorption. This result is not unexpected, as activation of a basolateral G_K would increase the driving force for Na+ entry across the apical membrane. In support of this, addition of mucosal UTP induces an initial rise in I_{sc}, which can be attributed to an increase in Na+ absorption (12, 37), and UTP activates a CTX-sensitive G_K that is likely identical to the K_{Ca} activated by 1-EBIO (hIK1).

We previously demonstrated that genistein stimulates Na+ absorption across HBE (12). Our present findings confirm this observation (Fig. 13), suggesting that genistein may have the unwanted effect of further increasing Na+ absorption across CF airway in vivo. The mechanism by which this stimulation occurs remains unclear, however. Genistein has been shown to directly activate CFTR in excised patch-clamp recordings (25, 54). Thus one possibility is that genistein modulates Na+ transport via an interaction with CFTR, as CFTR itself has been shown to negatively modulate ENaC activity (48). However, the CFTR opener NS004 has no effect on Na+ transport, which argues against this possibility. A second possibility is that genistein activates a basolateral G_K, thereby increasing the driving force for Na+ absorption. Indeed, direct activation of basolateral K_{Ca} by 1-EBIO or UTP (12, 37) stimulates Na+ absorption. We have found that genistein stimulates Cl− secretion across T84 monolayers in a 293B-sensitive manner (unpublished observations), suggesting that genistein not only activates CFTR but also basolateral G_K. Thus we speculate that genistein is activating a basolateral G_K to stimulate Na+ absorption across HBE. It should be noted that the magnitude of the increase in I_{sc} cannot be accounted for by Cl− secretion, as genistein has only modest effects on Cl− transport even in the presence of amiloride, where there would be an improved electrochemical driving force.

In summary, we demonstrate that both CFTR (NS004, 8-MOP, genistein) and K_{Ca} (1-EBIO) openers stimulate a sustained Cl− secretory response across HBE. In addition, all of these agonists also stimulate HCO_3− secretion, further suggesting that CFTR is the exit pathway for HCO_3− across the apical membrane. Importantly, we demonstrate that NS004 and genistein are capable of stimulating Cl− secretion across ΔF-HBE, suggesting that some ΔF508 CFTR escapes the degradative pathway to be expressed at the apical membrane, consistent with the results of Dupuit et al. (18, 19). Indeed, a mutation expected to produce only 2% of wt CFTR mRNA (2789 +5 G→A) results in expression of sufficient CFTR in the apical membrane to produce a pharmacological increase in anion secretion.

**Implications for Development of New Therapies for Airway Diseases**

Our results suggest pharmacological activators of CFTR or G_K may prove clinically useful in several settings. Recent data suggest that impaired Cl− secretion and increased Na+ absorption in CF airway epithelia impair mucociliary clearance (38), thereby mak-
ing the CF airway susceptible to infection. In CF, benzimidazolones, psoralens, or isoflavones may partially restore transepithelial Cl− secretion. By restoring Cl− permeability, these compounds may increase airway surface fluid volume and improve mucociliary clearance. Further studies are necessary to determine the effects of these compounds, alone and in combination with amiloride or compounds that augment trafficking of ΔF508 CFTR to the membrane (5, 60), on both Cl− and Na+ transport in vivo.

Mucociliary clearance is impaired in several other airway diseases, including chronic bronchitis and bronchiectasis (3, 53). Studies have suggested that β-adrenergic agonists and UTP improve mucociliary clearance in these diseases (39, 42, 59), presumably via increases in mucus secretion, ciliary beat frequency, and/or Cl− secretion. Because β-agonists and methylxanthines are bronchodilators, it is difficult to determine the contribution of fluid secretion to their favorable clinical effects. However, as demonstrated in this report, benzimidazolones, psoralens, and genistein augment Cl− secretion in human airway epithelia and may therefore be useful compounds for treatment of chronic bronchitis, bronchiectasis, and other diseases in which impaired mucociliary clearance contributes to airway obstruction. In support of this, recent data in normal volunteers indicate that chloroxazone, a skeletal muscle relaxant that is structurally related to 1-EBIO, and genistein, each stimulate Cl− secretion in human nasal epithelium (26, 46). Safety and dose-response studies to determine the effect of these compounds on mucociliary clearance and airways obstruction will be necessary to determine their utility as adjunctive therapies for patients with chronic bronchitis and bronchiectasis.

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