The dual phosphodiesterase 3 and 4 inhibitor RPL554 stimulates CFTR and ciliary beating in primary cultures of bronchial epithelia

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Turner MJ, Matthes E, Billet A, Ferguson AJ, Thomas DY, Randell SH, Ostrowski LE, Abbott-Banner K, Hanrahan JW. The dual phosphodiesterase 3 and 4 inhibitor RPL554 stimulates CFTR and ciliary beating in primary cultures of bronchial epithelia. *Am J Physiol Lung Cell Mol Physiol* 310: L59–L70, 2016. First published November 6, 2015; doi:10.1152/ajplung.00324.2015.—Cystic fibrosis (CF), a genetic disease caused by mutations in the CFTR gene, is a life-limiting disease characterized by chronic bacterial airway infection and severe inflammation. Some CFTR mutants have reduced responsiveness to cAMP/PKA signaling; hence, pharmacological agents that elevate intracellular cAMP are potentially useful for the treatment of CF. By inhibiting cAMP breakdown, phosphodiesterase (PDE) inhibitors stimulate CFTR in vitro and in vivo. Here, we demonstrate that PDE inhibition by RPL554, a drug that has been shown to cause bronchodilation in asthma and chronic obstructive pulmonary disease (COPD) patients, stimulates CFTR-dependent ion secretion across bronchial epithelial cells isolated from patients carrying the *R117H*/*F508del* CF genotype. RPL554-induced CFTR activity was further increased by the potentiator VX-770, suggesting an additional benefit by the drug combination. RPL554 also increased cilia beat frequency in primary human bronchial epithelial cells. The results indicate RPL554 may increase mucociliary clearance through stimulation of CFTR and increasing ciliary beat frequency and thus could provide a novel therapeutic option for CF.

cystic fibrosis; CFTR; phosphodiesterase; RPL554; ciliary beating

THE COMMON, AUTOSOMAL RECESSIVE disease cystic fibrosis (CF) results from mutations to the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) (52), a plasma membrane anion channel. Defective CFTR-dependent anion transport leads to a host of pathologies that affect the pancreas, small intestine, sweat glands, and airways (5, 34, 35, 49, 50, 64). In the airways, reduced secretion contributes to the accumulation of thick, sticky mucus and impaired mucociliary clearance, which in turn leads to bacterial colonization and chronic inflammation (41, 60). CFTR is tightly regulated by the cAMP/PKA signaling pathway (3, 12, 13, 17, 27, 54, 61), and therefore, pharmacological agents that elevate intracellular cAMP concentration ($[cAMP]_i$) have been suggested as potential therapeutic options for the treatment of CF (31). Cyclic nucleotide phosphodiesterases (PDEs) terminate signaling by hydrolyzing cyclic nucleotides (7). The PDE superfamily includes 11 gene families and over 30 isozymes, each with multiple splice variants that together comprise almost 100 different variants (42). Expression and localization of PDE isoenzymes are cell and tissue specific, and many PDEs are often expressed in specific microdomains that allow for highly organized spatiotemporal cyclic nucleotide signaling. For detailed reviews of PDEs, see Conti and Beavo (14), Francis et al. (21), and Maurice et al. (42).

PDE3 and PDE4 have been shown to regulate CFTR in primary airway epithelial cells and in epithelial cell lines. PDE4A5, PDE4C1, PDE4D2, and PDE4D3 expression was demonstrated in primary human airway epithelial cells (22), and PDE3 and PDE4 inhibitors have been shown to elevate $[cAMP]_i$ in airway epithelia, when added alone (12) or in combination with activators of adenylyl cyclase (22, 65). Furthermore, both PDE3 and PDE4 inhibitors have been shown to activate CFTR-dependent anion secretion across the human airway epithelial cell line Calu-3 (12, 48) while Barnes et al. (4) demonstrated increased CFTR channel activity when apical membrane patches excised from Calu-3 cells were exposed to a PDE4 inhibitor. CFTR can interact physically with PDE4D (38) and PDE3A (48), and exposure to a PDE3A inhibitor leads to clustering of CFTR and PDE3A into membrane microdomains and augments CFTR channel function (48). These results suggest that inhibitors of PDE3 and PDE4 could potentially be useful therapeutics for CF.

More recently, Blanchard et al. (6) demonstrated that the PDE4 inhibitor rolipram, when used in combination with CFTR potentiators and correctors, stimulates CFTR activity in primary human bronchial epithelial (HBE) cells homozygous for the most common CF mutation *F508del* (10). In addition to their CFTR-activating properties, PDE3 and 4 inhibitors also have beneficial anti-inflammatory and bronchodilator effects, which would provide additional benefits to CF patients. Di Paola et al. (19) demonstrated PDE3 inhibition significantly reduced tissue inflammation and the release of proinflammatory cytokines in a rat model of myocardial inflammation

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while PDE4 inhibition has also been shown to reduce the release of proinflammatory cytokines from a number of cell types, including airway epithelial cells, as well as suppressing recruitment of inflammatory cells to the airways (24–26, 37, 40, 45, 68).

Of further interest, there is increasing evidence to suggest that dual inhibition of PDE3 and PDE4 can have additive or synergistic anti-inflammatory, bronchodilator, and CFTR- activating effects (1, 39, 44, 48) suggesting that targeting both PDEs may be of greater benefit than inhibiting one alone. RPL554 is a "first in class" inhaled dual PDE3/4 inhibitor that has significant bronchodilator and anti-inflammatory activity in clinical trials (20). RPL554 has been demonstrated to induce sustained relaxant effects on contractile responses induced by spasmogens (histamine and carbachol) and electrical field stimulation in isolated airway tissue (human bronchial and guinea pig tracheal preparations). The degree of bronchial wall relaxation was greater than in tissue treated only with a β_2-agonist or anti-muscarinics, even when those agents were administered at maximal concentrations (9). Interestingly, RPL554 also interacts in an additive or synergistic manner with β_2-agonists or anti-muscarinics, respectively, when administered in combination in in vitro and in vivo experimental model systems (8, 30). The fact that RPL554 inhibits PDEs involved in CFTR regulation, as well as possessing anti-inflammatory and bronchodilator properties, suggests it could afford therapeutic benefit in CF patients. PDE inhibitors may also act on airway cilia since their motility is also controlled by cAMP/PKA (2, 18, 55, 63) probably through phosphorylation of dynein light chain in the axoneme (36) Indeed, the PDE4-selective inhibitor Roflimulast N-oxide increased ciliary beat frequency (CBF) in HBE (43).

The purpose of this study was to examine the effect of RPL554 on CFTR function in cell lines and primary HBE from CF patients with *R117H/F508del* and *F508del/F508del* genotypes. *R117H* reduces single channel conductance and open probability but does not affect trafficking to the plasma membrane or activation by cAMP/PKA (11, 57, 62). *F508del* reduces open probability and nearly abolishes trafficking to the plasma membrane without altering single channel conductance (10, 15). We also examined the effect of RPL554 on ciliary beating. Here we show that RPL554 stimulates the activity of wild-type (WT) and *R117H* CFTR, and this is primarily due to inhibition of PDE4. RPL554 also increases CBF primarily through inhibition of PDE4. These results suggest RPL554 as an attractive therapeutic option for CF and reveal novel mechanisms by which inhaled RPL554 may improve lung function in chronic obstructive pulmonary disease (COPD) and asthma patients.

METHODS

Culture of Chinese hamster ovary cells. The Chinese hamster ovary cell line CHO, expressing WT CFTR (61), was cultured in minimum essential media (MEM) containing 5% fetal bovine serum and the selecting drug methotrexate (100 mM) for 2 days. Cells were grown in T_{75} flasks (Corning), incubated at 37°C in humidified air containing 5% CO_2-95% O_2, and passaged every 7 days using trypsin/EDTA. For automated patch-clamp experiment, CHO were subcultured in 25-cm^2 plates and used at 80% confluence (~1 million cells).

Culture of CFBE cells. The HBE cell line CFBE was cultured in MEM supplemented with 10% (vol/vol) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml puromycin as a selection agent. Cells were grown in T_{75} flasks (Corning), incubated at 37°C in humidified air containing 5% CO_2-95% O_2, and passaged every 7 days using trypsin/EDTA. 80,000 cells were seeded onto collagen-coated 6.5-mm Corning Costar 0.4-μm pore, polyester membrane inserts and kept submerged 48 h until cells had formed a highly resistive monolayer with transepithelial electrical resistance (TEER) >400 Ω/cm^2 as measured using an epithelial volt-ohmmeter (World Precision Instruments). The apical medium was then removed and cells were maintained at the air-liquid interface (ALI) for another 7 days before being studied.

Isolation and culture of primary HBE cells. Human lung tissues were procured under the auspices of Institutional Review Board-approved protocols at McGill University and the University of North Carolina. Three lung specimens unused for transplantation were obtained from non-CF individuals, and three *F508del/F508del* specimens and three *R117H/F508del* specimens were procured after lung transplantation. The non-CF and *F508del/F508del* cells were from the CF Canada Primary Airway Cell Biobank at the McGill CF Translational Research Centre (CFTRc). *R117H/F508del* cells were from the Univeristy of North Carolina CF Center Tissue Procurement and Cell Culture Core. Isolation, culture, and differentiation of pHBE cells were adapted from procedures previously described by Fulcher et al. (23). Briefly, airway epithelial cells were isolated from bronchial tissue by enzyme digestion and cultured in bronchial epithelial growth medium on type I collagen-coated plastic flasks (Vitrogen 100, PureCol; Advanced BioMatrix) and then trypsinized, counted, and cryopreserved. Cells were seeded onto collagen coated 6.5-mm Costar 0.4-μm pore, polyester membrane inserts (Corning) and grown under submerged conditions for 4 days before the apical media were removed and the cells were allowed to differentiate at an ALI for a minimum of 21 days before use. The isolation and growth media were complemented with antibiotics selected according to recent patient microbiology reports. However, only penicillin and streptomycin were added to the ALI cultures.

RNA extraction and quantitative real-time PCR. Total cellular RNA was extracted and purified using the RNase Easy Mini Kit (Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. For reverse transcription, 500 ng RNA were incubated with 4 μl VILO Mastermix (Life Technologies, Burlington, ON, Canada) in a reaction volume of 20 μl for 1 h at 42°C and for 5 min at 85°C. Then, 250 ng cDNA, 10 μl TaqMan Fast Advanced Mastermix, and 1 μl TaqMan Gene Expression Assay primers were added to the wells of a MicroAmp EnduraPlate Optical 96-Well Fast Reaction Plate in a reaction volume of 20 μl. The quantitative PCR reaction consisted of 20 s at 95°C and then 40 cycles of 95°C (1 s) and 60°C (20 s) and was carried out in a QuantStudio 7 Flex Real-Time PCR system. $\Delta\Delta C_T$ analysis was carried out using QuantStudio 7 Flex Real-Time PCR system software in which the expression of each PDE gene was normalized to expression of GAPDH within that sample. Analysis of the standard curves made for each primer revealed primer efficiency to be >90% for each primer used.

Patch-clamp experiments. The effects of RPL554 on CFTR currents were measured using an automated patch-clamp technique. CHO cells were detached with Detachin (Genlantis, San Diego, CA) centrifuged for 5 min at 1,000 rpm, resuspended in MEM (without serum), and used immediately on the Qpatch 16X system (Sophion Bioscience). The holding potential was set to −40 mV during the entire experiment, and two voltage protocols were used to measured whole cell CFTR current. To control for the effect of various drug or concentration and test the absence of leak, a single depolarization from −40 at 0 mV was applied. After 5 min, the current/voltage (*I–V*) relationship was determined by pulsing from −40 mV between −80 and +80 mV in 20-mV increments. Experiments were conducted with single-hole Qplate at room temperature.

Short-circuit current measurements. Primary HBE cells were grown on collagen-coated polyester membrane inserts (6.5-mm Corn-

ing Costar 0.4-μm pore) and cultured in serum- and antibiotic-free medium with 20 μl medium applied to the apical membrane 24 h before study. Cells were mounted into modified Ussing chambers (Physiological Instruments, San Diego, CA) containing 5 ml saline, which was continuously gassed with 5% CO_2-95% O_2. Monolayers were clamped at 0 mV using a Multichannel Voltage-Current Clamp (Physiological Instruments) and currents recorded using a Powerlab 8SP (AD Instruments) and analyzed using LabChart 7.0 software. Transepithelial resistance (R_{te}) was monitored by applying a 10-mV pulse (duration: 2 s) every 30 s and calculating resistance by Ohm's law.

Measurements of CBF. Primary HBE cells were grown on 12-mm collagen-coated Millicell 0.4-μm pore inserts and cultures were visualized using a ×20 phase objective on a Nikon TE2000 microscope, and high-speed videos (60 fps) were recorded from 5 separate fields of each culture. CBF was determined from the videos using the whole field analysis option within the SAVA software package (58), and the average of the five measurements was used as the baseline CBF for that culture. Cultures were then treated with pharmacological agents and CBF was again determined from the average of five measurements taken ~1, 5, and 30 min after treatment. Data were analyzed as both the absolute change in CBF at each time point and the change in CBF relative to baseline. Each experiment was repeated at least three times with cells from three different donors ($n \geq 9$).

Solutions and reagents. All reagents were purchased from Sigma-Aldrich apart from $CFTR_{inh}$ 172 (Cystic Fibrosis Foundation Therapeutics), RPL554 (Verona Pharma, London, UK), and TNF-α (R&D Systems, Minneapolis, MN). Gases were purchased from MEGS Speciality Gases (Montreal, Canada). For patch-clamp recordings, the external bath solution contained the following (in mM): 145 NaCl, 4 CsCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 tetradecyl sulfate (TES) and titrated to pH 7.4. The intrapipette solution contained the following (in mM): 113 L-aspartic acid, 113 CsOH, 27 CsCl, 1 NaCl, 1 MgCl2, 1 ethyleneglycoltetraacetic acid, 1 TES, and 3 MgATP and titrated to pH 7.2. For short-circuit current (I_{sc}) measurements, the basolateral saline solution consisted of the following (in mM): 115 NaCl, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, and 10 D-glucose, while the apical saline solution consisted of the following (in mM): 1.2 NaCl, 115 Na-gluconate, 25 $NaHCO_3$, 1.2 $MgCl_2$, 4 $CaCl_2$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, and 10 D-glucose.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software. Student's *t*-test, one-way ANOVA (with Tukey's multiple comparison posttest), or two-way ANOVA (with Bonferroni posttest) was carried out where applicable to determine statistical significance between measurements. $P < 0.05$ was considered statistically significant.

RESULTS

RPL554 does not activate CFTR directly but potentiates forskolin-stimulated CFTR currents in CHO cells. To first gain insight into possible direct effects of RPL554, whole cell currents were recorded in CHO cells expressing CFTR. No activation was observed with RPL554 alone, indicating it does not act directly on CFTR and does not elevate cAMP significantly in CHO cells, which might be due to low basal flux through the cAMP pathway or insensitivity of endogenous PDEs to RPL554 (Fig. 1). Forskolin (10 μM) stimulated currents of 35.6 ± 15.2 pA/pF at +40 mV ($P < 0.001$ vs. unstimulated cells; $n = 3$; Fig. 1*A*), which was significantly reduced to 6.19 ± 2.70 pA/pF by $CFTR_{inh}$ 172 ($P < 0.05$; $n = 3$), demonstrating CFTR was active in these cells. However. RPL554 potentiated CFTR activity when cells were stimulated with a submaximal concentration of forskolin. Two micromoles of forskolin alone stimulated a CFTR-dependent current of 10.19 ± 0.81 pA/pF at +40 mV ($n = 4$), and this was dose dependently increased by RPL554, which reached statistical significance at 0.3 μM RPL554 (Fig. 1*B*). At the highest concentration of RPL554 tested (10 μM), CFTR-dependent currents were 103.34 ± 13.13 pA/pF at +40 mV ($n = 13$; $P < 0.001$ vs. forskolin alone; Fig. 1*B*) meaning 10 μM RPL554 had potentiated forskolin-stimulated CFTR activity ~10 fold. Therefore, although RPL554 failed to stimulate CFTR activity alone, the fact it could enhance forksolin-stimulated CFTR activity to such an extent indicates its potential to enhance CFTR activity.

RPL554 acts as a CFTR activator in WT CFBE cells. We next examined whether RPL554 was able to activate WT CFTR in the human airway epithelial cell line CFBE. RPL554 dose dependently increased CFTR-dependent I_{sc} with the highest concentration of RPL554 tested (10 μM) increasing I_{sc} by 49.8 ± 7.8 μA cm^{-2} ($n = 4$). For comparison this was 57.6 ± 3.8% of the stimulation produced by a maximal (10 μM) concentration of forskolin (86.5 ± 12.0 μA/cm^2; $n = 5$; Fig. 2). Therefore, these data suggested that RPL554, via inhibition of PDE3 and/or PDE4, raised $[cAMP]_i$ levels sufficiently to activate CFTR-dependent transepithelial anion secretion to levels that are comparable with those produced by forskolin. Thus

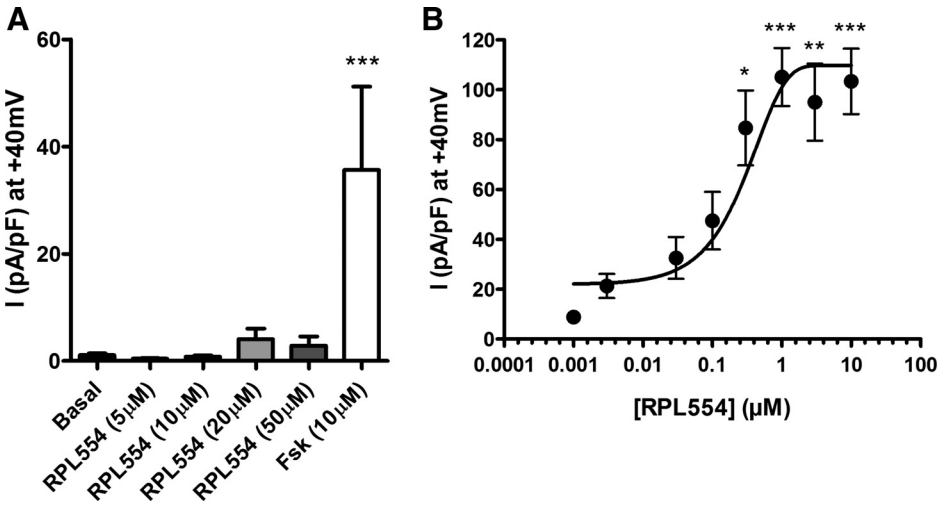

Fig. 1. RPL554 does not stimulate cystic fibrosis transmembrane conductance regulator (CFTR) directly but potentiates forskolin-stimulated CFTR current in Chinese hamster ovary (CHO) cells. CFTR-dependent currents in CHO cells expressing CFTR were measured using the broken patch, whole cell patch configuration. *A*: CFTR-dependent current obtained at +40 mV in response to stimulation of cells with RPL554 or forskolin (Fsk). Data represent means ± SE; $P < 0.001$; $n = 3$–19. *B*: increase in CFTR-dependent currents obtained at +40 mV by increasing concentrations of RPL554 after cells had been prestimulated with forskolin (2 μM). Data represent means ± SE; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$;$n = 6$–17.

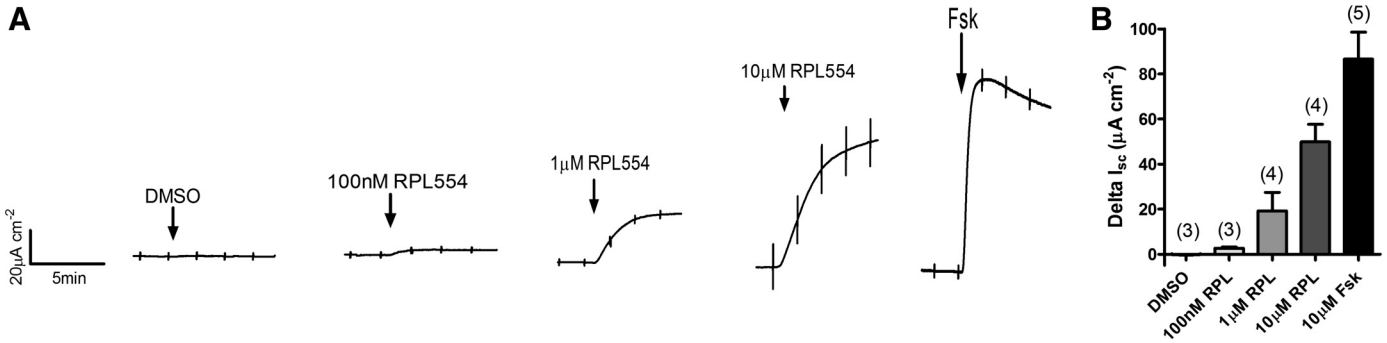

Fig. 2. RPL554 stimulates CFTR-dependent short-circuit current (I_{sc}) in wild-type (WT) CFBE cells. CFBE cells expressing WT CFTR were grown at air-liquid interface (ALI) for 7 days before I_{sc} measurements were made in an Ussing chamber. *A*: example experiments in which I_{sc} was measured in response to increasing concentrations of RPL554 (100 nM to 10 μM) and forskolin (10 μM). *B*: summary of the data. Data represent means ± SE; *n* numbers are displayed in parenthesis.

RPL554 activates robust secretion by this human airway cell line in the absence of other secretagogues.

PDE expression is altered in CF pHBE cells compared with WT pHBE cells. RPL554 is a dual PDE3/4 inhibitor; therefore, we examined the expression of these PDE isoenzymes, together with other PDE isoforms as well as examining whether their expression was altered in CF. Expression of a subset of eight known PDE isoforms was assessed in HBE cells from three non-CF donors, three patients homozygous for *F508del*, and three patients carrying *R117H/F508del*. By assessing the relative expression of each PDE gene tested when normalizing to the expression of GAPDH, we were able to get an indication of which PDE genes were most abundantly expressed in HBE cells. As shown in Fig. 3*A*, PDE4D was expressed in relatively high abundance in WT cells, which is unsurprising given the body of evidence implicating PDE4D in CFTR regulation in human airway epithelia. However, there was a very low expression of PDE3A when normalized to GAPDH expression across all three genotypes, indicating PDE3A is expressed at very low levels in HBE cells relative to other PDE isoforms. An unexpected finding was the abundance of PDE7A and PDE8A expression in all three genotypes, raising the question as to their role in cyclic nucleotide signaling in human airway epithelia. When normalizing the PDE gene expression relative to the WT patients, we observed a 5.1 ± 2.3-fold upregulation of PDE3A and a 3.9 ± 1.9-fold upregulation of PDE4D in *F508del/F508del* cells compared with WT ($P < 0.001$ vs. WT; $n = 3$ for each; Fig. 3*B*), which suggests a loss of CFTR at the cell surface may modulate expression of proteins involved in CFTR regulation.

PDE inhibition does not stimulate CFTR-dependent transepithelial ion transport in HBE F508del/F508del cells but increases the response to VX-770. To assess whether *1*) RPL554 had similar CFTR stimulatory effects in HBE cells as those observed in CFBE cells and *2*) RPL554 was able to potentiate the activity of mutant CFTR, the effect of RPL554 was tested in *F508del/F508del* HBE cells. To gain insight into the mechanism of action, the effects of RPL554 were also compared with those of the PDE3-selective inhibitor milrinone and the PDE4-selective inhibitor rolipram. Cells were pretreated for 24 h with VX-809 (1 μM) and VX-770 (100 nM) to mimic the current treatment for *F508del/F508del* patients. A submaximal concentration of forskolin (2 μM) stimulated an increase in I_{sc} of 2.9 ± 0.3 μA cm^{-2} ($n = 25$; Fig. 4). However, subsequent exposure to specific PDE inhibitors did not further enhance I_{sc} and actually caused a small decrease in CFTR-dependent I_{sc}. PDE-stimulated increases in I_{sc} after forskolin stimulation were −0.3 ± 0.1, −0.3 ± 0.1, −1.0 ± 0.4, and −0.1 ± 0.1 μA/cm^2 by milrinone (10 μM), rolipram (10 μM), milrinone + rolipram, and RPL554 (10 μM), respec-

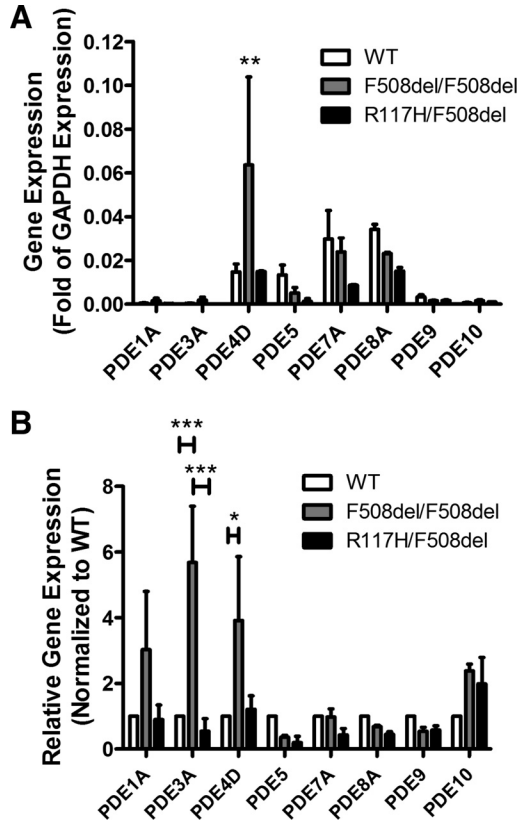

Fig. 3. Phosphodiesterase (PDE) isoform expression in human bronchial epithelial (HBE) cells. HBE cells were isolated and cultured as described in METHODS and gene expression was assessed using quantitative PCR. *A*: expression of each PDE gene when normalized to the expression of GAPDH in each sample. $^{**}P < 0.01$, significant difference between WT and *F508del/F508del* cells. *B*: expression of each PDE gene, normalized to the expression of GAPDH in each sample, and normalized to the expression levels seen in WT cells. $^{*}P < 0.05$, $^{***}P < 0.001$, significant difference between WT and *F508del/F508del* HBE cells. Data represent means ± SE; $n = 3$ different patients for each genotype.

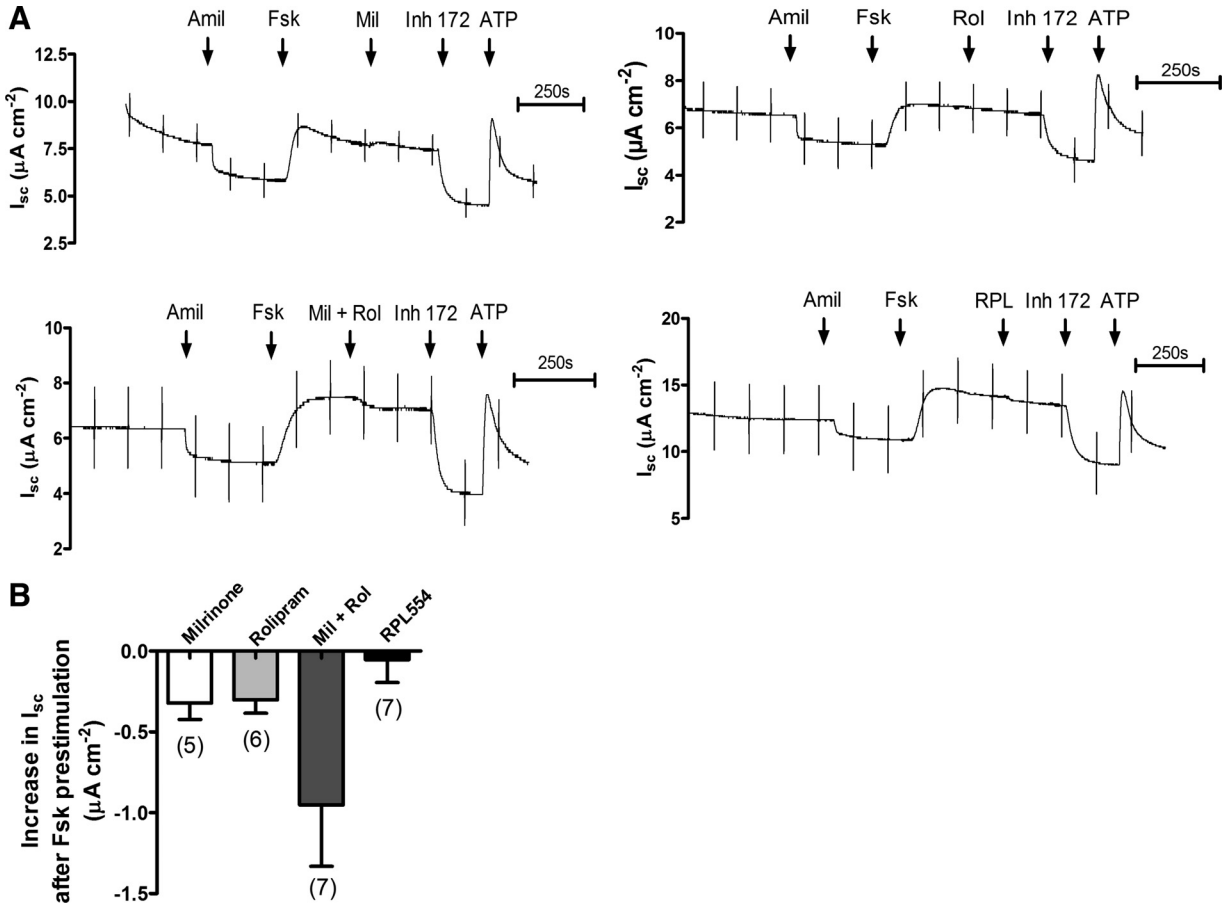

Fig. 4. PDE3/4 inhibitors do not potentiate forskolin-stimulated CFTR-dependent I_{sc} in HBE *F508del/F508del* cells. *F508del/F508del* HBE cells were isolated and cultured as described in METHODS and I_{sc} measurements were made in Ussing chambers. Cells were treated with the CFTR corrector VX-809 (3 μM) and the CFTR potentiator VX-770 (100 nM) for 24 h before study. *A*: representative experiments in which I_{sc} was measured in response to treatment with forskolin (2 μM) followed by either the PDE3 inhibitor milrinone (Mil; 10 μM apical; *top left*), the PDE4 inhibitor rolipram (Rol; 10 μM apical; *top right*), milrinone and rolipram (Mil + Rol; *bottom left*), and RPL554 (10 μM apical; *bottom right*). Cells were first treated with apical amiloride (Amil; 100 μM) and the current was inhibited by $CFTR_{inh}$ 172 (Inh 172; 10 μM apical) while cells maintained functionality, demonstrated by a response to apical ATP (100 μM). *B*: summary of the increase in I_{sc} in response to PDE inhibition after 2 μM forskolin prestimulation. Data represent means ± SE; *n* numbers are displayed in parenthesis.

tively (n = 5–7; Fig. 4). These results suggest that *F508del* CFTR at the membrane was fully activated by forskolin following rescue by the corrector VX-809 and chronic potentiation by VX-770, and thus any subsequent increases in cAMP were unable to further stimulate CFTR-dependent anion transport. We also investigated whether PDE inhibition could stimulate *F508del* CFTR in noncorrected cells (i.e., cells not treated for 24 h with VX-809 or VX-770). In these conditions, forskolin (2 μM) stimulated a negligible increase in I_{sc} of 0.05 ± 0.02 μA/cm^2 (n = 12), while subsequent stimulation of cells with either rolipram (10 μM) or RPL554 (10 μM) caused highly variable increases in I_{sc} that were not significantly different to DMSO controls (DMSO = 0.05 ± 0.03 μA/cm^2; rolipram = 0.13 ± 0.10 μA/cm^2; RPL554 = 0.03 ± 0.15 μA/cm^2; $P > 0.05$; n = 3–6; Fig. 5) These data demonstrate that *F508del* CFTR does not respond to cAMP stimulation in noncorrected cells. However, PDE inhibition was able to significantly increase the VX-770-stimulated increase in I_{sc} from 0.34 ± 0.13 μA/cm^2 in control cells to 0.74 ± 0.06 and 0.80 ± 0.05 μA/cm^2 in rolipram and RPL554-stimulated cells, respectively ($P < 0.05$ vs. control; n = 3–6; Fig, 5). Thus, although ineffective at stimulating *F508del* CFTR alone, PDE inhibitors can augment VX-770-dependent potentiation of *F508del* CFTR suggesting that RPL554 and VX-770 in combination might provide additional benefit to *F508del/F508del* patients.

RPL554 stimulates CFTR-dependent transepithelial ion transport in pHBE R117H/ΔF508 cells by inhibiting PDE4. Next, we explored the role of different PDEs in modulating the response of CF HBE cells (*R117H/F508del*) to cAMP stimulation. A submaximal concentration of forskolin (2 μM) stimulated an increase in I_{sc} of 1.4 ± 0.1 μA/cm^2 (n = 16; Fig. 6). Inhibiting basal PDE activity with either milrinone, rolipram, milrinone + rolipram, or RPL554 caused negligible increases in I_{sc} (0.1 ± 0.02, 0.5 ± 0.1, 0.1 ± 0.3, and 0.2 ± 0.2 μA/cm^2, respectively; n = 3–7; Fig. 7), suggesting that PDE3 and 4 inhibition alone did not elevate $[cAMP]_i$ sufficiently to stimulate *R117H* CFTR. However, when added after exposure to a submaximal concentration of forskolin, stimulation was observed with the specific PDE4 inhibitor rolipram (0.9 ± 0.2 μA/cm^2; $P < 0.01$; n = 6), a combination of specific PDE3 and PDE4 inhibitors (milrinone + rolipram; 0.7 ± 0.1 μA/cm^2; $P < 0.05$; n = 5), and with the dual PDE3/PDE4 inhibitor RPL554 (0.9 ± 0.2 μA/cm^2; $P < 0.01$; n = 9; Fig. 7). By

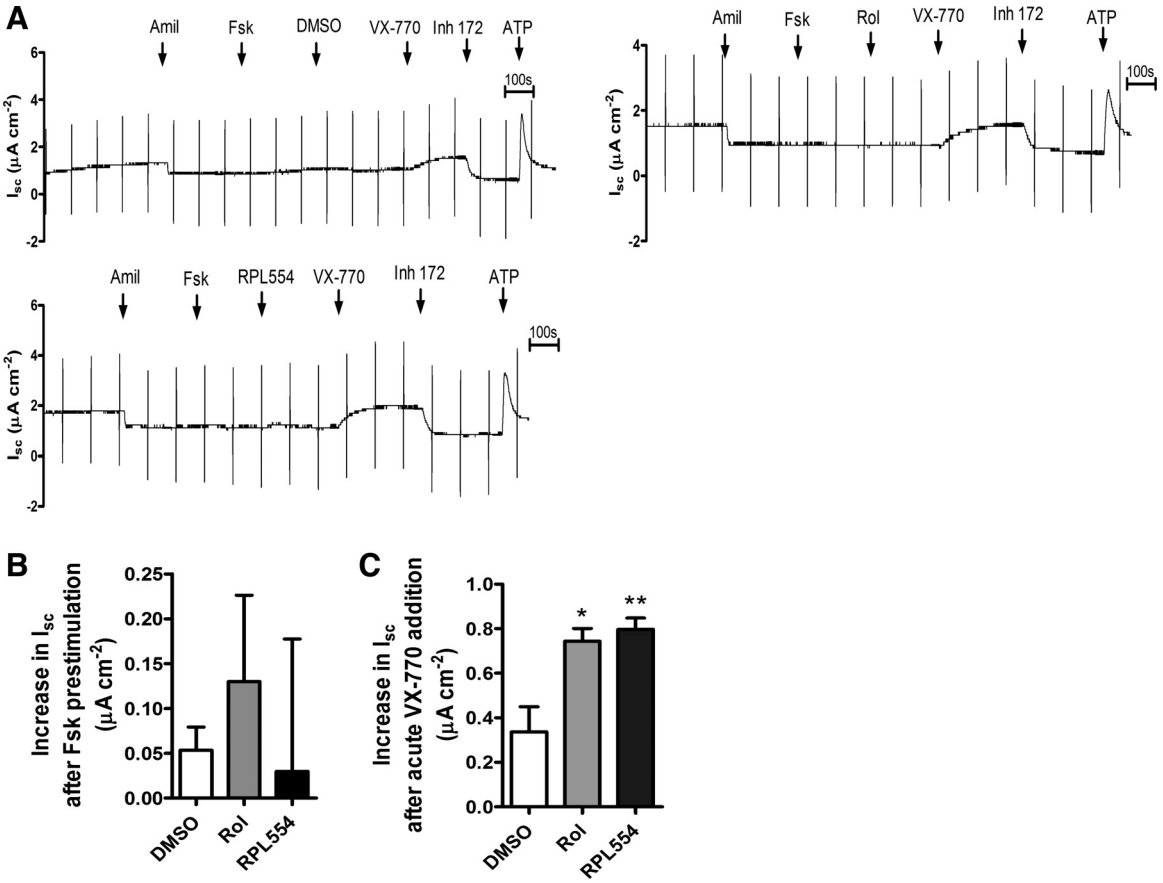

Fig. 5. PDE3/4 inhibitors do not stimulate noncorrected F508del CFTR but can potentiate the stimulation by acute VX-770. *F508del/F508del* HBE cells were isolated and cultured as described in METHODS and I_{sc} measurements were made in Ussing chambers. *A*: representative experiments in which I_{sc} was measured in response to treatment with forskolin (2 μM) followed by either DMSO (*top left*), the PDE4 inhibitor rolipram (10 μM apical; *top right*), or RPL554 (10 μM apical; *bottom left*). The response to acute VX-770 (100 nM apical) was also assessed. Cells were first treated with apical amiloride (100 μM) and the current was inhibited by $CFTR_{inh}$ 172 (10 μM apical) while cells maintained functionality, demonstrated by a response to apical ATP (100 μM). *B*: summary of increase in I_{sc} in response to PDE inhibition after 2 μM forskolin prestimulation. *C*: summary of the increase in I_{sc} in response to acute VX-770. $*P < 0.05$, $**P < 0.01$, significant effect of PDE inhibition vs. DMSO. Data represent means ± SE; $n = 3$ for DMSO and rolipram and $n = 6$ for RPL554.

contrast, the response to milrinone alone after submaximal forskolin stimulation was negligible (−0.03 ± 0.1 μA/cm^2; $n = 5$; Fig. 7) indicating that PDE3 has little role in regulating CFTR. Together, these results identify PDE4 as the primary regulator of CFTR-dependent transepithelial ion secretion in HBE *R117H* cells prestimulated with forskolin. Interestingly, when *R117H/F508del* HBE cells were pretreated with VX-809, forskolin-stimulated I_{sc} was enhanced but not the response to PDE inhibitors (Figs. 6 and 7). This suggests that CF cells have diminished regulation of the cAMP microdomain surrounding CFTR, which is not fully corrected by VX-809 and may be due to alterations in both the cytoskeleton (46) and the localization of PDE4.

PDE3 and 4 contribute to compartmentalization of cAMP signaling in CF airway epithelia. A well-established role for PDEs is to compartmentalize cAMP in a specific subcellular microdomain to allow for efficient spatiotemporal cAMP signaling to occur (4) although this role has been challenged with respect to CFTR regulation (46). To investigate the role of PDEs during global and localized elevations of cAMP, CF (*R117H/ΔF508*) HBE cells were stimulated with forskolin to increase [cAMP] throughout the cell, or apical adenosine, to increase cAMP preferentially in the subapical membrane microdomain. Forskolin stimulated a larger increase in CFTR-dependent I_{sc} (1.4 ± 0.1 μA/cm^2; $n = 16$), compared with that induced by apical adenosine (0.2 ± 0.2 μA/cm^2; $P < 0.001$; $n = 6$; Fig. 8*A*). However, the subsequent response to PDE inhibitors was similar in cells stimulated with either agonist. Milrinone + rolipram increased I_{sc} by 0.6 ± 0.1 and 0.8 ± 0.3 μA/cm^2 after pretreatment with forskolin and adenosine, respectively ($P > 0.05$; $n = 3$–9; Fig. 8*B*), and RPL554 increased I_{sc} by 0.9 ± 0.2 and 0.7 ± 0.2 μA/cm^2, respectively ($P > 0.05$; $n = 3$–9; Fig. 8*B*). Together, these data imply that PDE regulation occurs predominantly in the subapical cAMP compartment in HBE *R117H/F508del* cells.

VX-770 and RPL554 have additive effects on CFTR-dependent anion transport. Given that we have already shown that PDE inhibition enhances CFTR activity in *R117H/F508del* HBE cells, it was interesting to test the effect of PDE inhibition in combination with the clinically approved CFTR potentiator VX-770 (Kalydeco). To this end, *R117H/F508del* cells were stimulated with 2 μM forskolin and then treated with either 10 μM RPL554 followed by 100 nM VX-770 (Fig. 9*A*, *left*) or in reverse order; i.e., 100 nM VX-770 followed by 10 μM RPL554 (Fig. 9*A*, *right*). RPL554 further increased the forskolin-stimulated CFTR-dependent I_{sc} by 2.4 ± 0.2 μA/cm^2

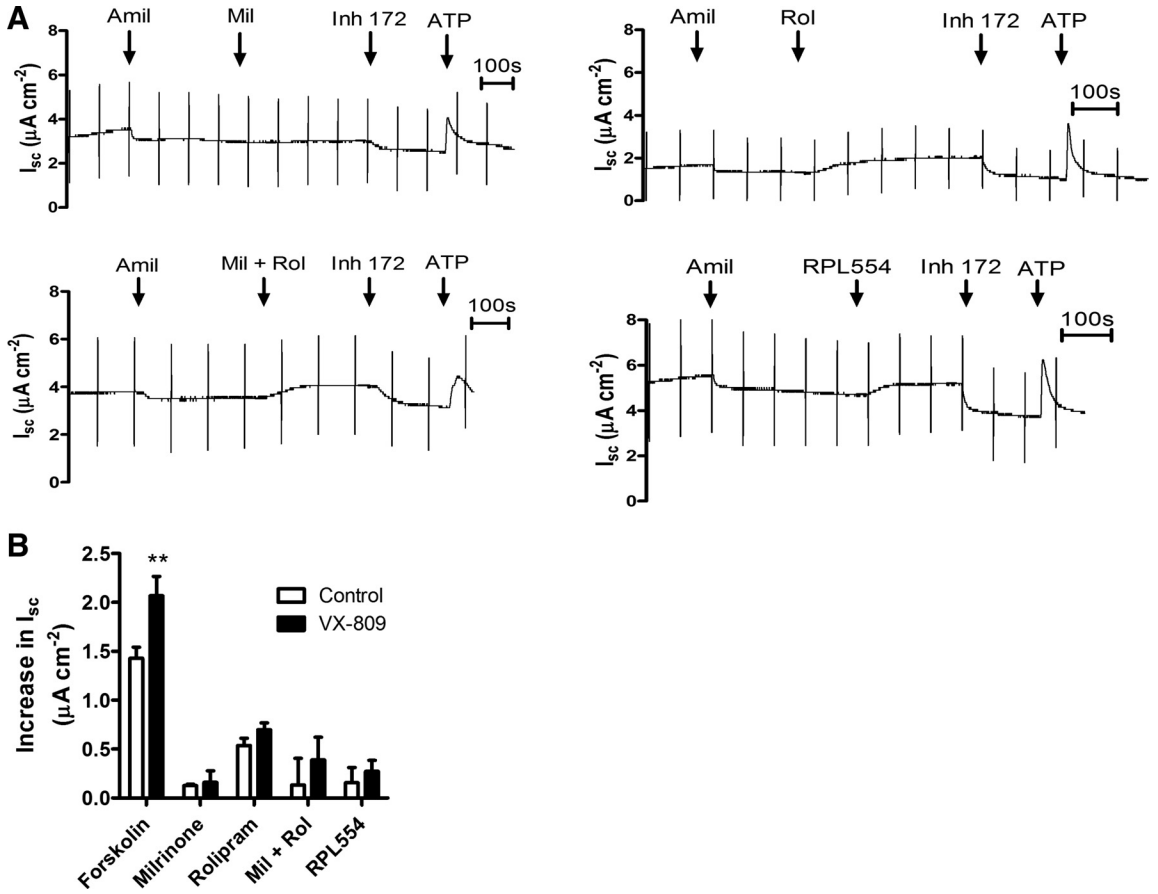

Fig. 6. PDE3/4 inhibitors alone stimulate negligible CFTR-dependent I_{sc} in *R117H/F508del* HBE cells. *R117H/F508del* HBE cells were isolated and cultured as described in METHODS and I_{sc} measurements were made in Ussing chambers. Cells were treated with DMSO or the CFTR corrector VX-809 (3 μM) for 24 h before study. *A*: representative experiments in which I_{sc} was measured in response to treatment with the PDE3 inhibitor milrinone (10 μM apical; *top left*), the PDE4 inhibitor rolipram (10 μM apical; *top right*), milrinone and rolipram (*bottom left*), and RPL554 (10 μM apical; *bottom right*). Cells were first treated with apical amiloride (100 μM) and the current was inhibited by $CFTR_{inh}$ 172 (10 μM apical) while cells maintained functionality, demonstrated by a response to apical ATP (100 μM). *B*: summary of the data and how PDE inhibition compares to stimulation with a submaximal dose of forskolin (2 μM) and the effect of 24 h pretreatment with VX-809. $^{**}P < 0.01$, significant effect of VX-809 in forskolin-stimulated cells. Data represent means ± SE; *n* numbers are displayed in parenthesis.

($n = 4$), and the subsequent addition of VX-770 caused a further increase of 6.2 ± 0.3 μA/cm^2. The net increase in CFTR-dependent I_{sc} under these conditions (8.6 ± 0.6 μA/cm^2) was significantly greater than when either agonist was added alone ($P < 0.01$; $n = 3$; Fig. 9*B*). Adding VX-770 after forskolin stimulation caused I_{sc} to increase by 4.3 ± 0.5 μA/cm^2 ($n = 17$) However, subsequent addition of RPL554 failed to increase CFTR activity further and the total stimulation was only about half that when RPL554 was added first. These data indicate that larger responses are obtained with these agents in combination; however, the enhanced response depends on the order of exposure. Thus a combination therapy involving VX-770 and RPL554 could provide additional benefits to *R117H/F508del* patients although this may depend on their order of administration and pharmacokinetics.

Exposure of R117H/F508del HBE cells to proinflammatory cytokines does not alter the effect of PDE inhibition on CFTR-dependent I_{sc}. In an attempt to mimic the pathophysiological state of CF airways, *R117H/F508* HBE cells were treated with the proinflammatory cytokine TNF-α (1 ng/ml) for 24 h before assessing the CFTR-dependent I_{sc} in response to PDE3/4 inhibition. Pretreatment with TNF-α did not significantly alter the response to PDE inhibition after prestimulation with submaximal forskolin (2 μM); milrinone stimulation was negligible in control cells (0.3 ± 0.2 μA/cm^2; $n = 3$) and in TNF-α-treated cells (−0.01 ± 0.02 μA/cm^2; $P > 0.05$ vs. control; $n = 3$; Fig. 10), demonstrating that PDE3 inhibition does not modulate CFTR activity, even when cells are in a proinflammatory state. Similarly, in cells that had been pretreated with TNF-α and stimulated with forskolin, there was no increase ($P > 0.05$; $n = 3$) in the I_{sc} response to rolipram (2.6 ± 0.1 vs. 1.9 ± 0.4 μA/cm^2), milrinone + rolipram (2.0 ± 0.4 vs. 2.2 ± 0.1 μA/cm^2), and RPL554 (1.5 ± 0.4 vs. 1.7 ± 0.3 μA/cm^2) when compared with control cells (Fig. 10).

RPL554 increases CBF in HBE cells. In addition to CFTR-dependent anion secretion, mucociliary clearance depends on ciliary beating, a process that is also regulated by cAMP signaling (2, 18, 36, 55, 63). We therefore measured CBF in WT HBE cells to assess whether it is increased by RPL554. Treatment of cells with RPL554 for 30 min significantly increased CBF by 2.6 ± 0.8% at 1 min posttreatment compared with time-matched DMSO controls ($P < 0.05$; $n = 11$), 8.1 ± 1.2% at 5 min posttreatment ($P < 0.001$; $n = 11$) and 10.4 ± 1.8% at 30 min posttreatment ($P < 0.001$; $n = 11$; Fig. 11).

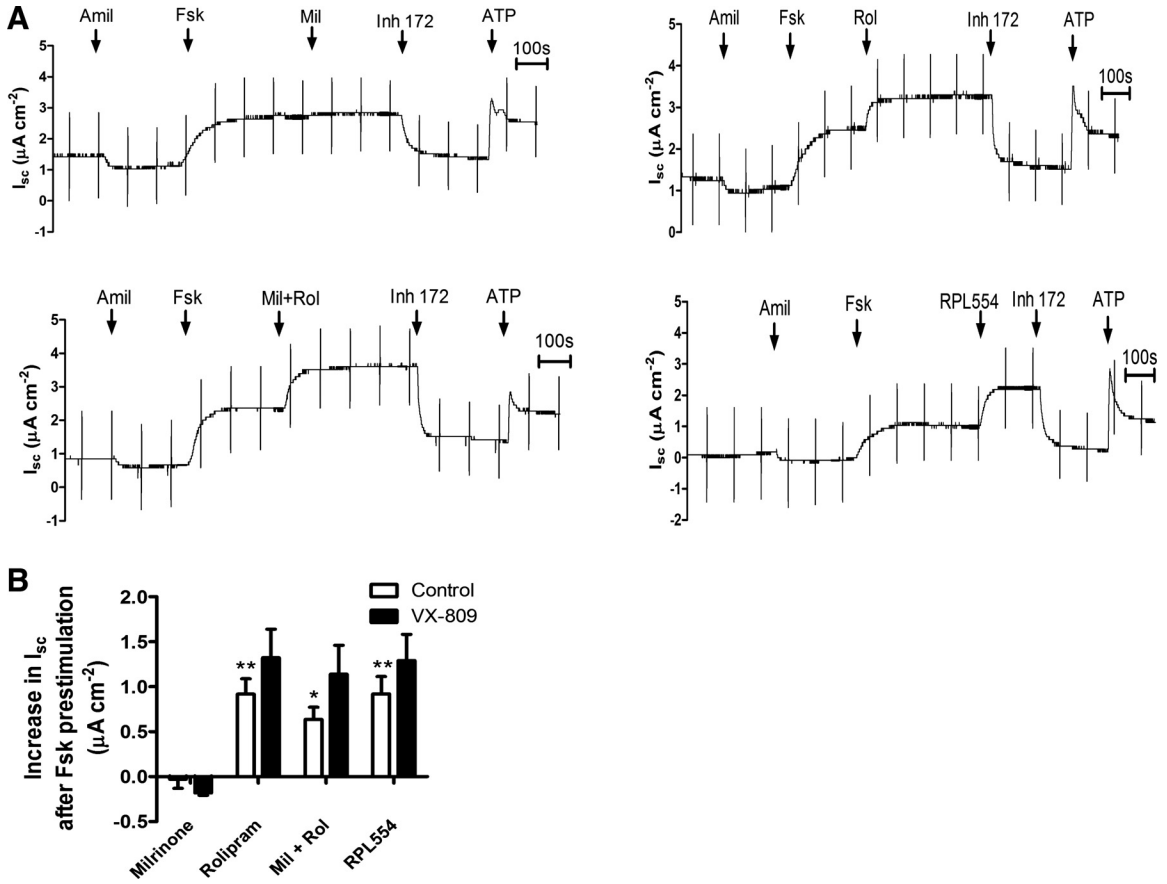

Fig. 7. PDE4 inhibition stimulates CFTR-dependent I_{sc} in *R117H/F508del* HBE cells after prestimulation with forskolin. *R117H/F508del* HBE cells were isolated and cultured as described in METHODS and I_{sc} measurements were made in Ussing chambers. Cells were treated with DMSO or the CFTR corrector VX-809 (3 μM) for 24 h before study. *A*: representative experiments in which I_{sc} was measured in response to treatment with the PDE3 inhibitor milrinone (10 μM apical; *top left*), the PDE4 inhibitor rolipram (10 μM apical; *top right*), milrinone and rolipram (*bottom left*), and RPL554 (10 μM apical; *bottom right*). Cells were first treated with apical amiloride (100 μM) and then stimulated with submaximal concentration of apical forskolin (2 μM). The resulting I_{sc} increase was inhibited by $CFTR_{inh}$ 172 (10 μM apical) while cells maintained functionality, demonstrated by a response to apical ATP (100 μM). *B*: summary of data and how PDE inhibition compares to stimulation with a submaximal dose of forskolin (2 μM) and the effect of 24 h pretreatment with VX-809. $*P < 0.05$, $**P < 0.01$, significant effect of PDE inhibitor vs. milrinone alone. Data represent means ± SE; *n* numbers are displayed in parenthesis.

The effect of RPL554 was almost identical to that of the selective PDE4 inhibitor roflumilast (Fig. 11), providing evidence that RPL554 increases ciliary beating and therefore is predicted to promote mucociliary clearance in both WT and CF patients.

DISCUSSION

By inhibiting phosphodiesterase-dependent breakdown of cAMP, PDE3, and PDE4 inhibitors have previously been shown to stimulate CFTR in human airway epithelial cells, suggesting they could be a therapeutic option for the treatment of CF. Here, we investigated the effect of RPL544, a dual PDE3/4 inhibitor that has bronchodilator effects in asthma and COPD patients (20), for its ability to restore CFTR function in several cell types, including primary HBE. Firstly, we demonstrated that although RPL554 was not able to stimulate CFTR in CHO cells, indicative of low basal adenylyl cyclase activity, it was able to potentiate CFTR activity when cells were stimulated by a submaximal concentration of forskolin (Fig. 1). These findings imply that inhibition of PDE3 and 4 in CHO cells enhanced the increase in $[cAMP]_i$ after forskolin stimulation sufficiently to further stimulate CFTR. By contrast, RPL554 did stimulate CFTR in the bronchial epithelial cell line CFBE under basal conditions in a dose-dependent manner (Fig. 2). Although the highest concentration of RPL554 tested was less potent than forskolin, PDE3/4 inhibition was clearly able to raise $[cAMP]_i$ sufficiently to activate CFTR. These results with CFBE cells are similar to those reported for the airway cell line Calu-3 in which PDE3 and PDE4 inhibitors induced 10–85% of the forskolin response (12). More specifically, the fact that PDE4D has been shown to be involved in cAMP compartmentalization (4) suggests that the effects of RPL554 may be mediated by increased cAMP levels within the CFTR microdomain that depends on transmembrane adenylyl cyclase (tmAC) activity. The distinct effects of RPL554 in CHO and CFBE cells suggest CFBE cells have much higher basal tmAC activity and consequentially cAMP turnover and highlight the cell-type specific effects of RPL554.

We next compared the effects of the dual PDE3/4 inhibitor RPL554 with those of the PDE3-selective inhibitor (milrinone) and the PDE4-selective inhibitor (rolipram). CFTR-dependent anion transport was measured in primary HBE cells from CF patients carrying *F508del/ΔF508del* or *R117H/F508del* alleles. Quantitative PCR results revealed upregulation of PDE3

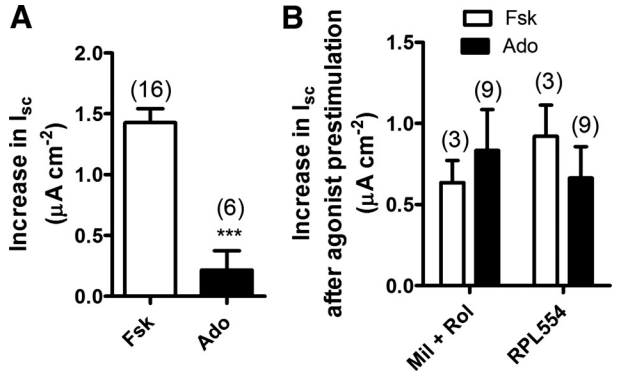

Fig. 8. PDE3/4 contributes to cAMP compartmentalization in *R117H/F508del* HBE cells. *R117H/F508del* HBE cells were isolated and cultured as described in METHODS and I_{sc} measurements were made in Ussing chambers. *A*: increase in CFTR-dependent I_{sc} after cells were stimulated with either forskolin (2 μM) or apical adenosine (Ado; 10 μM). *B*: further I_{sc} increase after subsequent inhibition of PDE3/4 using either milrinone (10 μM) + rolipram (10 μM) or RPL554 (10 μM) was also assessed. ***$P < 0.001$, significant difference between forskolin and adenosine. Data represent means ± SE; *n* numbers are displayed in parenthesis.

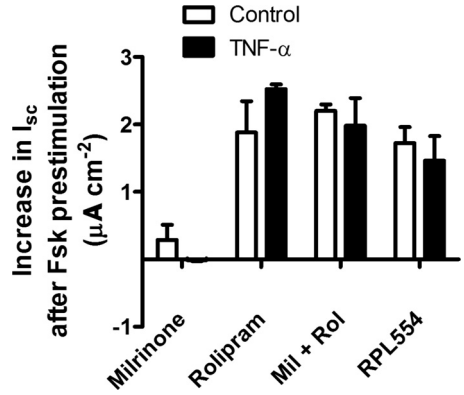

Fig. 10. TNF-α treatment does not modulate PDE inhibitor-stimulated CFTR-dependent I_{sc} in *R117H/F508del* HBE cells. *R117H/F508del* HBE cells were isolated and cultured as described in METHODS and I_{sc} measurements were made in Ussing chambers. Cells were treated with PBS (control) or TNF-α (1 ng/ml) 24 h before the study and CFTR-dependent I_{sc} was measured in response to milrinone (10 μM), rolipram (10 μM), milrinone + rolipram, and RPL554 (10 μM) after cells were prestimulated with forskolin (2 μM). Data represent means ± SE; $n = 3–4$.

and PDE4D expression in CF cells when compared with WT cells, but the overall level of PDE3 expression was very low compared with PDE4D and, interestingly, PDE7A and PDE8A (Fig. 3). In *F508del/F508del* cells pretreated with VX-770 and VX-809, forskolin stimulated CFTR-dependent I_{sc}; however, PDE inhibitors did not cause further I_{sc} increases suggesting that *F508del* CFTR was maximally activated after forskolin stimulation (Fig. 4). This is perhaps surprising when one considers that the gene expression of PDE3 and PDE4D was upregulated in *F508del/F508del* cells; however, it is worth noting that mRNA abundance may not strictly be proportional to protein expression. In noncorrected *F508del/F508del* cells, forskolin failed to increase CFTR-dependent I_{sc} and subsequent PDE inhibition also did not activate CFTR, demonstrating the importance of VX-809 and VX-770 therapy for these patients. However, in the presence of PDE inhibitors, VX-770-stimulated I_{sc} was significantly enhanced compared with control cells, suggesting PDE inhibitors can stimulate CFTR after potentiation by VX-770 (Fig. 5). In *R117H/F508del* cells, PDE inhibition alone failed to stimulate CFTR-dependent I_{sc}, although rolipram, milrinone + rolipram, and RPL554 all increased I_{sc} when cells were prestimulated with forskolin. The lack of effect of milrinone as well as similar I_{sc} stimulations elicited by the PDE4-selective inhibitor rolipram and the dual PDE3/4 inhibitor RPL554 suggests PDE4 inhibition mediates most stimulation of *R117H* CFTR (Figs. 6–7). This is consistent with the very low PDE3 gene expression measured in these cells and agrees with other studies that have demonstrated PDE4 activity to be significantly higher than PDE3 activity in HBE cells (16) and PDE4 to be the predominant regulator of CFTR in human airway epithelia (4, 6). However, PDE3 inhibition stimulates CFTR in Calu-3 cells (12, 48) raising the possibility that PDE regulation of CFTR differs in airway gland and surface epithelia. The negative effects of PDE inhibition in *F508del/F508del* cells compared with the positive effects in *R117H/F508del* cells show that the CFTR response to PDE inhibitors in *R117H/F508del* cells must be due to the *R117H* allele. This highlights a difference in the regulation of *R117H* CFTR and *F508del* CFTR since *R117H* CFTR is not

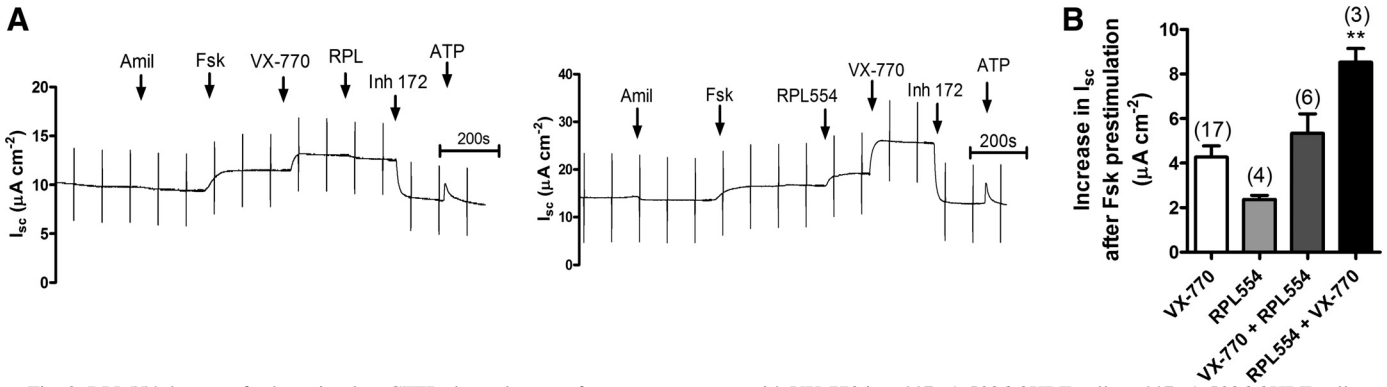

Fig. 9. RPL554 does not further stimulate CFTR-dependent I_{sc} after acute treatment with VX-770 in *R117H/F508del* HBE cells. *R117H/F508del* HBE cells were isolated and cultured as described in METHODS and I_{sc} measurements were made in Ussing chambers. *A*: representative experiments in which cells were stimulated with forskolin and then treated with either VX-770 (100 nM) followed by RPL554 (10 μM; *left*) or RPL554 followed by VX-770 (*right*). *B*: increase in I_{sc} after treatment with each drug either alone or in combination. **$P < 0.01$, significant effect of RPL554− VX-770 vs. either agonist alone. Data represent means− SE; *n* numbers are displayed in parenthesis.

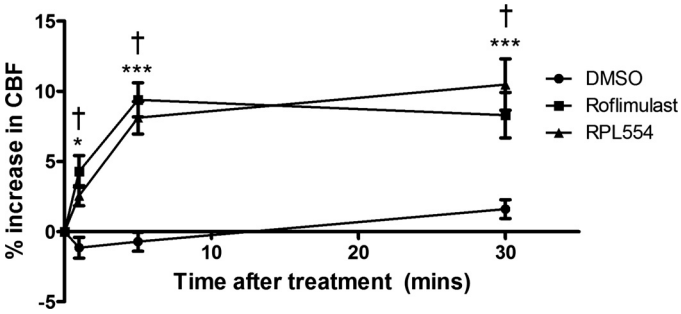

Fig. 11. RPL554 increases ciliary beat frequency (CBF) in HBE cells. WT HBE cells were isolated and cultured as described in METHODS and CBF was measured using video microscopy. Cells were treated with DMSO (control), roflumilast (1 μM), or RPL554 (10 μM) and the CBF was measured 1, 5, and 30 min posttreatment. $*P < 0.05$, $***P < 0.001$, significant effect of RPL554 vs. DMSO; $\dagger P < 0.001$, significant effect of roflumilast vs. DMSO. Data represent means ± SE of 3–4 repeat experiments from 3 different patients (n = 9–11).

fully stimulated by forskolin and can be further modulated by local changes in cAMP, in contrast to rescued *F508del* CFTR.

When responses to submaximal forskolin and apical adenosine were compared in *R117H/F508del* HBE cells, forskolin stimulated higher CFTR-dependent anion transport than adenosine (Fig. 8*A*). This parallels earlier findings in Calu-3 cells using the patch-clamp technique (28). RPL554 and the combination of milrinone + rolipram caused similar increases in I_{sc} regardless of whether the cells had been prestimulated with forskolin or adenosine, indicating that PDE3/4 inhibition abolished the compartmentalized nature of cAMP signaling produced by adenosine receptor stimulation. PDE4-dependent compartmentalization of cAMP in Calu-3 airway epithelial cells was reported by Barnes et al. (4), who showed that inhibitors of PDE4, but not PDE3, can abolish the lateral confinement of cAMP signals that are induced by adenosine receptor activation. The PDE4 inhibitor rolipram also stimulated CFTR when added to excised patches and augmented adenosine-stimulated CFTR activity to levels observed during forskolin stimulation. By contrast, Kelley et al. (32) showed that CFTR in Calu-3 is strongly activated by the PDE3 inhibitor milrinone and weakly stimulated by rolipram. Similar results were reported by Penmatsa et al. (48), who found that the PDE3 inhibitor cilostazol stimulated CFTR more strongly than rolipram in Calu-3 cells. They also demonstrated an interaction between CFP-PDE3A and YFP-CFTR in HEK cells using fluorescence resonance energy transfer (FRET) and coimmunoprecipitated HA-PDE3A with Flag-CFTR. Although some findings remain controversial, they reinforce the importance of PDE activity in regulating local cAMP levels in HBE cells.

Considering that RPL554 increases forskolin-stimulated CFTR channel activity, it was interesting to examine its effects in the presence of VX-770, a clinically prescribed CFTR potentiator. VX-770 binds directly to CFTR to increase channel gating and thus potentiates its activity after stimulation with cAMP agonists (29, 66). We observed that VX-770 further enhanced CFTR activity in cells treated with forskolin and RPL554 (Fig. 9). Thus these findings demonstrate CFTR activity can still be increased by CFTR potentiators, even after forskolin and RPL554 stimulation, highlighting that gating limits CFTR activity in the presence of cAMP and that forskolin and RPL554 did not stimulate CFTR to its maximal activity in *R117H/ΔF508* cells. However, when cells were stimulated with forskolin and then potentiated with VX-770, subsequent addition of RPL554 failed to further activate CFTR. This indicates that forskolin-induced phosphorylation of CFTR is sufficient for a maximal response to VX-770, and once VX-770 has potentiated CFTR further increases in $[cAMP]_i$, due to PDE inhibition have no effect on CFTR activity. Interestingly, when RPL554 was added first followed by VX-770, the net I_{sc} increase was significantly greater than when either agonist was added alone or combined in the reverse order. This implies that combining these two drugs provides the highest level of CFTR stimulation although this will likely depend on the pharmacokinetics of both compounds and require administration of RPL554 before VX-770. It is important to note that, in these studies, VX-770 was added acutely so it remains to be seen what would happen in the clinic under chronic dosing conditions.

CF airways are chronically inflamed, mediated by high levels of proinflammatory cytokines, including IL-8 and TNF-α (33, 51, 56, 59). Therefore, we examined whether CFTR-dependent anion transport in response to PDE inhibitors was altered in cells exposed to proinflammatory cytokines. However, 24-h treatment with TNF-α did not affect the response to PDE inhibition as only PDE4 inhibition stimulated CFTR-dependent I_{sc} in control and TNF-α-treated cells (Fig. 10). However, these data do show that the effects of RPL554 are not compromised by the presence of TNF-α and therefore support its potential therapeutic use in CF airways.

Mucociliary clearance depends on CFTR-driven fluid secretion and ciliary beating; therefore, we examined the effect of RPL554 on CBF. CBF was increased significantly, consistent with the regulation of CBF by cAMP signaling (Fig. 11). A similar increase in CBF was observed with roflumilast, which is in agreement with previous findings (43). The similar increases in CBF induced by roflumilast and RPL554 suggest the response was mediated by inhibition of PDE4. Although equivalent experiments were not yet performed using CF cells, since CBF is regulated by PKA-dependent phosphorylation of dynein light chains, the regulation of CBF is probably not CFTR dependent and should be similar in CF cells.

In summary, we have demonstrated for the first time that the PDE3/4 dual inhibitor RPL554 stimulates CFTR in well-differentiated HBE cells from *R117H/F508del* and therefore could provide therapeutic benefits to patients carrying at least one *R117H* allele. The mechanism of action apparently involves exclusively PDE4 and is additive with the effect of VX-770; therefore, it may further enhance CFTR activity in combination with VX-770. RPL554 also increased CBF in well-differentiated HBE cells, which again was seemingly mediated by PDE4 inhibition. These findings imply that RPL554 would serve to improve lung function in CF patients by *1*) stimulating CFTR-dependent anion transport allowing for mucus hydration and *2*) increasing CBF to enhance mucociliary clearance. Furthermore, CFTR activation causes relaxation of rat and human airway smooth muscle (47, 53, 67); thus RPL554 activation of CFTR may contribute to clinical improvement in COPD patients through improved mucus clearance by the epithelium or through airway smooth muscle relaxation and subsequent bronchodilation.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

M.J.T., L.E.O., K.A.-B., and J.W.H. conception and design of research; M.J.T., E.M., A.B., and A.J.F. performed experiments; M.J.T., E.M., A.B., and A.J.F. analyzed data; M.J.T., L.E.O., K.A.-B., and J.W.H. interpreted results of experiments; M.J.T., E.M., and A.B. prepared figures; M.J.T. drafted manuscript; M.J.T., S.H.R., L.E.O., K.A.-B., and J.W.H. edited and revised manuscript; M.J.T., E.M., A.B., A.J.F., D.Y.T., S.H.R., L.E.O., K.A.-B., and J.W.H. approved final version of manuscript.

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