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

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Running title: RPL554 stimulates CFTR in primary bronchial epithelia
Abstract:

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 signalling, 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 which has been shown to cause bronchodilation in asthma and 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 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.

Keywords: Cystic fibrosis, CFTR, phosphodiesterase, RPL554, ciliary beating
Introduction:

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 affects 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 signalling pathway (3, 12, 13, 17, 27, 54, 61) and therefore pharmacological agents that elevate [cAMP]$_i$ have been suggested as potential therapeutic options for the treatment of CF (31). Cyclic nucleotide phosphodiesterases (PDEs) terminate signalling 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 is cell and tissue specific and many PDEs are often expressed in specific microdomains which allow for highly organized spatio-temporal cyclic nucleotide signalling. 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 pro-inflammatory cytokines in a rat model of
myocardial inflammation while PDE4 inhibition has also been shown to reduce the release of
pro-inflammatory 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
which 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 (EFS) 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 \( \beta_2 \) 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 $\beta_2$ 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 human bronchial epithelia (43).

The purpose of this study was to examine the effect of RPL554 on CFTR function in cell lines and primary human bronchial epithelia (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 WT and $R117H$ CFTR, and this is primarily due to inhibition of PDE4. RPL554 also increases ciliary beat frequency 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 COPD and asthma patients.

**Methods:**

*Culture of CHO cells:* The Chinese hamster ovary cell line, CHO, expressing wild-type CFTR (61) was cultured in Minimum Essential Media (MEM) containing 5% fetal bovine serum and the selecting drug methotrexate (100mM) for 2 days. Cells were grown in
Culture of CFBE cells: The human bronchial epithelial cell line, CFBE, was cultured in Minimum Essential Media (MEM) supplemented with 10% (v/v) FCS, 100U/ml penicillin, 100μg/ml streptomycin and 2μg/ml puromycin as a selection agent. Cells were grown in T75 flasks (Corning), incubated at 37˚C in humidified air containing 5% CO2/95% O2 and passaged every 7 days using trypsin/EDTA. 80,000 cells were seeded onto collagen coated 6.5mm Corning Costar® 0.4μm pore, polyester membrane inserts and kept submerged 48h until cells had formed a highly resistive monolayer with transepithelial electrical resistance (TEER) > 400Ω cm² as measured using an epithelial volt-ohmmeter (World Precision Instruments, FL, USA). The apical medium was then removed and cells were maintained at the air-liquid interface for another 7 days before being studied.

Isolation and culture of primary human bronchial epithelial 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 3 F508del/F508del specimens and 3 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 UNC CF Center Tissue Procurement and Cell Culture Core. Isolation, culture, and differentiation of pHBE cells was 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), then trypsinized, counted, and cryopreserved. Cells were
seeded onto collagen coated 6.5mm Costar® 0.4μm pore, polyester membrane inserts (Corning) and grown under submerged conditions for 4 days before the apical media was removed and the cells were allowed to differentiate at an air–liquid interface (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) according to the manufacturer’s instructions. For reverse transcription, 500ng RNA was incubated with 4 μl VILO Mastermix (Life Technologies, Burlington, ON) in a reaction volume of 20μl for 1 h at 42°C and for 5 min at 85°C. 250ng cDNA, 10μl TaqMan® Fast Advanced Mastermix and 1μl TaqMan® Gene Expression Assay primers was added to the wells of a MicroAmp® EnduraPlate™ Optical 96-Well Fast Reaction Plate in a reaction volume of 20μl. The qPCR reaction consisted of 20 secs at 95°C, then 40 cycles of 95°C (1 sec) and 60°C (20 secs) and was carried out in a QuantStudio™ 7 Flex Real-Time PCR system. ΔΔCT 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: 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 mins at 1000 rpm, resuspended in MEM (without serum) and used immediately on the Qpatch 16X system (Sophion Bioscience, Denmark). 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
collection and test the absence of leak, a single depolarization from -40 mV at 0 mV was
applied. After 5 mins, 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 (6.5mm Corning Costar® 0.4μm pore) and cultured in
serum- and antibiotic-free medium with 20 μl medium applied to the apical membrane 24 h
prior to study. Cells were mounted into modified Ussing chambers (Physiological
Instruments, San Diego, CA) containing 5 ml saline, which was continuously gassed with 5%
CO2/95% O2. Monolayers were clamped at 0 mV using a Multichannel Voltage-Current
Clamp (Physiological Instruments) and currents recorded using a Powerlab 8SP (AD
Instruments, Colarado, USA) and analysed using LabChart 7.0 software. Transepithelial
resistance (Rte) was monitored by applying a 10 mV pulse (duration 2 sec) every 30 sec and
calculating resistance by Ohm’s law.

Measurements of cilia beat frequency: Primary HBE cells were grown on 12mm
collagen coated Millicell® 0.4μm pore inserts and cultures were visualized using a 20x phase
objective on a Nikon TE2000 microscope and high-speed videos (60fps) were recorded from
5 separate fields of each culture. Ciliary beat frequency (CBF) was determined from the
videos using the whole-field analysis option within the SAVA software package (58) and the
average of the 5 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
5 measurements taken approximately 1, 5 and 30 minutes after treatment. Data was 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 3x with cells from 3 different donors (n ≥9).

**Solutions and reagents:** All reagents were purchased from Sigma-Aldrich apart from CFTRinh 172 (Cystic Fibrosis Foundation Therapeutics), RPL554 (Verona Pharma plc, London, UK) and TNF-α (R & D Systems, Minneapolis, USA). Gases were purchased from MEGS Speciality Gases (Montreal, Canada). For patch-clamp recordings, the external bath solution contained (in mM) 145 NaCl, 4 CsCl, 1 CaCl2, 1 MgCl2, 10 glucose and 10 tetradecyl sulfate (TES) and titrated to pH 7.4. The intrapipette solution contained (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 Isc measurements, the basolateral saline solution consisted of (in mM) 115 NaCl, 25 NaHCO3, 1.2 MgCl2, 1.2 CaCl2, 2.4 KH2PO4, 1.24 K2HPO4, and 10 D-Glucose while the apical saline solution consisted of (in mM) 1.2 NaCl, 115 Na-Gluconate, 25 NaHCO3, 1.2 MgCl2, 4 CaCl2, 2.4 KH2PO4, 1.24 K2HPO4, 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 post-test) or two way ANOVA (with Bonferroni post-test) were carried out where applicable to determine statistical significance between measurements. A p value of <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. 1A) which was significantly reduced to 6.19 ± 2.70 pA/pF by CFTRinh (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. 2 μM 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. 1B). 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. 1B) 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 forskolin-stimulated CFTR activity to such an extent indicates its potential to enhance CFTR activity.

**RPL554 acts as a CFTR activator in CFBEwt cells:** We next examined whether RPL554 was able to activate wtCFTR 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 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 to 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 figure 3A, 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 signalling 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 to WT (p<0.001 vs. WT; n=3 for each; Fig. 3B) 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 (i) RPL554 had similar CFTR stimulatory effects in HBE cells as those observed in CFBE cells and (ii) 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 pre-treated for 24 hours 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 Isc of 2.9 ± 0.3 μA cm⁻² (n=25; Fig. 4). However, subsequent exposure to specific PDE inhibitors did not further enhance Isc and actually caused a small decrease in CFTR-dependent Isc. PDE-stimulated increases in Isc after forskolin stimulation were -0.3 ± 0.1 μA cm⁻², -0.3 ± 0.1 μA cm⁻², -1.0 ± 0.4 μA cm⁻² and -0.1 ± 0.1 μA cm⁻² by milrinone (10 μM), rolipram (10 μM), milrinone + rolipram and RPL554 (10 μM) respectively (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 non-corrected cells (i.e. cells not treated for 24 hours with VX-809 or VX-770). In these conditions, forskolin (2 μM) stimulated a negligible increase in Isc 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 Isc 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 non-corrected cells. However, PDE inhibition was able to significantly increase the VX-770-stimulated increase in Isc from 0.34 ± 0.13 μA cm⁻² in control cells to 0.74 ± 0.06 μA cm⁻² 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 μA cm$^{-2}$, 0.5 ± 0.1 μA cm$^{-2}$, 0.1 ± 0.3 μA cm$^{-2}$ 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] 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 contrast, the response to milrinone alone after sub-maximal forskolin stimulation was negligible (-0.03 ± 0.1μA 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 pre-treated 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.

PDE 3 and 4 contribute to compartmentalization of cAMP signalling 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 signalling 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 sub-apical membrane microdomain. Forskolin stimulated a larger increase in CFTR-dependent $I_{sc}$ (1.4 ± 0.1 μA cm$^{-2}$; $n$=16), compared to that induced by apical adenosine (0.2 ± 0.2 μA cm$^{-2}$; p<0.001; $n$=6; Fig 8A). 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 μA cm$^{-2}$ and 0.8 ± 0.3 μA cm$^{-2}$ after pre-treatment with forskolin and adenosine, respectively (p>0.05; $n$=3-9; Fig. 8B), and RPL554 increased $I_{sc}$ by 0.9 ± 0.2 μA cm$^{-2}$ and 0.7 ± 0.2 μA cm$^{-2}$, respectively (p>0.05; $n$=3-9; Fig. 8B). Together, these data imply that PDE regulation occurs predominantly in the sub-apical 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. 9A, left panel) or in reverse order; i.e. 100 nM VX-770 followed by 10 μM RPL554 (Fig. 9A, right panel). RPL554 further increased the forskolin-stimulated CFTR-dependent $I_{sc}$ by 2.4 ± 0.2 μA cm$^{-2}$ (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. 9B). 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 pro-inflammatory 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 pro-inflammatory cytokine TNF-α (1 ng/ml) for 24 h prior to assessing the CFTR-dependent I_{sc} in response to PDE3/4 inhibition. Pre-treatment with TNF-α did not significantly alter the response to PDE inhibition after pre-stimulation 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 pro-inflammatory state. Similarly, in cells that had been pre-treated 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 μA cm^{-2} vs. 1.9 ± 0.4 μA cm^{-2}), milrinone + rolipram (2.0 ± 0.4 μA cm^{-2} vs. 2.2 ± 0.1 μA cm^{-2}) and RPL554 (1.5 ± 0.4 μA cm^{-2} vs. 1.7 ± 0.3 μA cm^{-2}) when compared with control cells (Fig. 10).

*RPL554 increases ciliary beat frequency in HBE cells.* In addition to CFTR-dependent anion secretion, mucociliary clearance depends on ciliary beating - a process which is also regulated by cAMP signalling (2, 18, 36, 55, 63). We therefore measured ciliary beat frequency (CBF) in WT HBE cells to assess whether it is increased by RPL554. Treatment of cells with RPL554 for 30 mins significantly increased CBF by 2.6 ± 0.8% at 1 minute post treatment when compared to time-matched DMSO controls (p<0.05; n=11), 8.1 ± 1.2% at 5 mins post treatment (p<0.001; n=11) and 10.4 ± 1.8% at 30 mins post treatment (p<0.001; n=11; Fig. 11). 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 which has bronchodilator effects in asthma and COPD patients (20), for its ability to restore CFTR function in several cell types, including primary human bronchial epithelia. Firstly, we demonstrated that although RPL554 was not able to stimulate CFTR in CHO cells, indicative of low basal adenyl 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, given 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 which depends on transmembrane adenyl cyclase (tmAC) activity. The distinct effects of RPL554 in CHO and CFBE cells suggest CFBE cells have much higher basal tmAC activity and consequently 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. qPCR results revealed upregulation of PDE3 and PDE4D expression in CF cells when compared with WT cells but the overall level of PDE3 expression was very low compared to PDE4D and, interestingly, PDE7A and PDE8A (Fig. 3). In F508del/F508del cells pre-treated 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 were upregulated in F508del/F508del cells; however, it is worth noting that mRNA abundance may not strictly be proportional to protein expression. In non-corrected 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 to 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 which 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 $F508\text{del}/F508\text{del}$ cells compared to the positive effects in $R117\text{H}/F508\text{del}$ cells show that the CFTR response to PDE inhibitors in $R117\text{H}/F508\text{del}$ cells must be due to the $R117\text{H}$ allele. This highlights a difference in the regulation of $R117\text{H}$ CFTR and $F508\text{del}$ CFTR since $R117\text{H}$ CFTR is not fully stimulated by forskolin and can be further modulated by local changes in cAMP, in contrast to rescued $F508\text{del}$ CFTR.

When responses to submaximal forskolin and apical adenosine were compared in $R117\text{H}/F508\text{del}$ HBE cells, forskolin stimulated higher CFTR-dependent anion transport than adenosine (Fig 8A). 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 pre-stimulated with forskolin or adenosine, indicating that PDE3/4 inhibition abolished the compartmentalized nature of cAMP signalling 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 FRET, and co-immunoprecipitated 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], 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 pro-inflammatory 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 pro-inflammatory 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 supports 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 ciliary beat frequency. CBF was increased significantly, consistent with the regulation of CBF by cAMP signalling (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 (i) stimulating CFTR-dependent anion transport allowing for mucus hydration and (ii) 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.

Acknowledgements:

We acknowledge the UK CF trust for the funding they have contributed towards these studies and we also thank Bob Bridges (Rosalind Franklin Univ.) and CFFT Inc. for CFTRinh172.
References:


Figure Legends:

Figure 1: RPL554 does not stimulate CFTR directly but potentiates forskolin-stimulated CFTR current in CHO cells: CFTR-dependent currents in CHO cells expressing CFTR were measured using the broken-patch, whole-cell patch configuration. (A) displays the CFTR-dependent current obtained at +40 mV in response to stimulation of cells
with RPL554 or forskolin. Data represents mean ± S.E.M., n=3-19. (B) displays the increase in CFTR-dependent currents obtained at +40 mV by increasing concentrations of RPL554 after cells had been pre-stimulated with forskolin (2 μM). Data represents mean ± S.E.M., n=6-17.

**Figure 2: RPL554 stimulates CFTR-dependent I_{sc} in CFBEwt cells:** CFBE cells expressing WT CFTR were grown at ALI for 7 days before I_{sc} measurements were made in an Ussing Chamber. (A) displays example experiments in which I_{sc} was measured in response to increasing concentrations of RPL554 (100 nM – 10 μM) and forskolin (10 μM). (B) summarizes the data. Data represents mean ± S.E.M.; n numbers displayed in parenthesis.

**Figure 3: Phosphodiesterase isoform expression in HBE cells:** HBE cells were isolated and cultured as described in Methods and gene expression was assessed using qPCR. (A) displays the expression of each PDE gene when normalized to the expression of GAPDH in each sample. ** = significant difference between WT and F508del/F508del cells (p<0.01). (B) displays the expression of each PDE gene, normalized to the expression of GAPDH in each sample, and normalized to the expression levels seen in WT cells. * = significant difference between WT and F508del/F508del HBE cells (p<0.05; ** = p<0.001). Data represents mean ± S.E.M; n=3 different patients for each genotype.

**Figure 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 (3μM) and the CFTR potentiator VX-770 (100nM) for 24 hours prior to study. (A) shows representative experiments in which I_{sc} was measured in response to treatment with forskolin (2μM) 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 CFTRinh 172 (Inh 172; 10 μM apical) while cells maintained functionality, demonstrated by a response to apical ATP (100 μM). (B) summarizes the increase in Isc in response to PDE inhibition after 2 μM forskolin prestimulation. Data represents mean ± S.E.M.; n numbers are displayed in parenthesis.

**Figure 5: PDE3/4 inhibitors do not stimulate non-corrected F508del CFTR but can potentiate the stimulation by acute VX-770:** F508del/F508del HBE cells were isolated and cultured as described in Methods and Isc measurements were made in Ussing chambers. (A) shows representative experiments in which Isc was measured in response to treatment with forskolin (2μM) followed by either DMSO (top left), the PDE4 inhibitor rolipram (Rol; 10 μM apical; top right) or RPL554 (10 μM apical; bottom left). The response to acute VX-770 (100nM apical) was also assessed. Cells were first treated with apical amiloride (Amil; 100 μM) and the current was inhibited by CFTRinh 172 (Inh 172; 10 μM apical) while cells maintained functionality, demonstrated by a response to apical ATP (100 μM). (B) summarizes the increase in Isc in response to PDE inhibition after 2 μM forskolin prestimulation and (C) summarizes the increase in Isc in response to acute VX-770. * = significant effect of PDE inhibition vs DMSO (p<0.05; ** = p<0.01). Data represents mean ± S.E.M.; n=3 for DMSO and rolipram and n=6 for RPL554.

**Figure 6: PDE3/4 inhibitors alone stimulate negligible CFTR-dependent Isc in R117H/F508del HBE cells:** R117H/F508del HBE cells were isolated and cultured as described in Methods and Isc measurements were made in Ussing Chambers. Cells were treated with DMSO or the CFTR corrector VX-809 (3μM) for 24 hours prior to study. (A)
shows representative experiments in which $I_{sc}$ was measured in response to treatment with 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) summarizes the data and shows how PDE inhibition compares to stimulation with a submaximal dose of forskolin (2μM) and the effect of 24 h pre-treatment with VX-809. * = significant effect of VX-809 in forskolin-stimulated cells (p<0.01). Data represents mean ± S.E.M.; n numbers are displayed in parenthesis.

**Figure 7: PDE4 inhibition stimulates CFTR-dependent $I_{sc}$ in R117H/F508del HBE cells after pre-stimulation 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 (3μM) for 24 hours prior to study. (A) shows representative experiments in which $I_{sc}$ was measured in response to treatment with 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 then stimulated with submaximal concentration of apical forskolin (Fsk; 2 μM). The resulting $I_{sc}$ increase 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) summarizes the data and shows how PDE inhibition compares to stimulation with a submaximal dose of forskolin (2 μM) and the effect of 24 h pre-treatment with VX-809. * = significant effect of PDE inhibitor vs. milrinone alone (p<0.05; ** = p<0.01). Data represents mean ± S.E.M.; n numbers displayed in parenthesis.
Figure 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) displays the increase in CFTR-dependent I_sc after cells were stimulated with either forskolin (2 μM) or apical adenosine (10 μM). (B) displays the 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. *** = significant difference between forskolin and adenosine (p<0.001). Data represents mean ± S.E.M.; n numbers displayed in parenthesis.

Figure 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) displays 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) displays the increase in I_sc after treatment with each drug either alone or in combination. ** = significant effect of RPL554 + VX-770 vs. either agonist alone (p<0.01). Data represents mean ± S.E.M.; n numbers displayed in parenthesis.

Figure 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 prior to 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 represents mean ± S.E.M.; n=3-4.
Figure 11: RPL554 increases ciliary beat frequency 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 min, 5 mins and 30 mins post treatment. * = significant effect of RPL554 vs. DMSO (p<0.05; *** = p<0.001); † = significant effect of roflumilast vs. DMSO (p<0.001) Data represents mean ± S.E.M. of 3-4 repeat experiments from 3 different patients (n=9-11).
Figure 1

A

B

A graph showing the current (pA/pF) at +40mV for different conditions: Basal, RPL554 (5nM), RPL554 (10μM), RPL554 (20μM), RPL554 (50μM), and Fak (10μM).

B

A graph showing the current (pA/pF) at +40mV as a function of [RPL554] (μM) with significant increases indicated by asterisks (*, **, ***).
Figure 2

A

[Graph showing the effects of DMSO, 100nM RPL554, 1μM RPL554, and 10μM RPL554 on current density over time.]

B

[Bar graph showing the increase in I_{sc} (μA cm^{-2}) with different treatments: DMSO, 100nM RPL, 1μM RPL, 10μM RPL, 10μM Fsk. Number of experiments indicated in parentheses.]
Figure 3

A

Gene Expression (Fold of GAPDH Expression)

B

Relative Gene Expression (Normalized to WT)
Figure 4

A

B
Figure 6

A

B

Increase in $I_{sc}$

Control

VX-809

Feokolin

Mirtorine

Rapipam

Mil + Rol

RPL564
Figure 7

(A) Graphs showing the change in $I_{sc}$ (uA cm$^2$) over time with various treatments: Amil, Fsk, Mil, Inh 172, and ATP. The graphs are repeated for different treatments including Mil + Rol, Inh 172, and ATP.

(B) Bar chart showing the increase in $I_{sc}$ after Fsk stimulation with different treatments: Control, VX-809, Naloxone, Rolipram, Mil + Rol, and RPL554.
Figure 8

A

B

Increase in $I_{sc}$

Increase in $I_{sc}$ after agonist pretreatment

(μA cm$^{-2}$)

Fsk
Ado

(16)

(6)

(3)

(9)

(9)

(9)

(3)

(3)

(9)

(3)

(9)
Figure 9

A

B

Increase in $I_{sc}$ after Fsk pre-stimulation (μA cm$^{-2}$)

<table>
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<th>Treatment</th>
<th>Increase (μA cm$^{-2}$)</th>
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<td>VX-770</td>
<td>2.5 ± 0.5</td>
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<tr>
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**p < 0.01
Figure 11