Regulatory domain phosphorylation to distinguish the mechanistic basis underlying acute CFTR modulators


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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial anion channel. Absent or defective CFTR causes abnormal ion transport, engendering organ dysfunction primarily in the lung, gastrointestinal tract, vas deferens, and sweat duct. As a member of the ATP-binding cassette (ABC) protein family, CFTR shares structural features with other ABC proteins, including the presence of two transmembrane domains (TMs) and two nucleotide-binding domains (NBDs). CFTR also possesses a regulatory domain (R-D), a unique feature among ABC proteins. Activation of CFTR anion transport requires phosphorylation of the R-D by PKA and PKC, as well as dimerization of the two NBDs, although the precise mechanism has not yet been described. Binding and hydrolysis of ATP at the NBD dimer interface is thought to induce a conformational change in the channel pore through interactions between the NBD and TM domains, allowing the passage of chloride ions and bicarbonate through the plasma membrane.

There are over 1,500 mutations of CFTR known to cause CF. Some of these mutations exclusively compromise activation of CFTR expressed at the cell surface. Patients with these types of CFTR mutations would be expected to benefit greatly from agents that restore activity, including direct activators of the channel or agents that augment endogenous regulatory mechanisms. Several compounds discovered in high-throughput screening (HTS) that increase CFTR activity on a rapid timescale (<30 min) have been proposed as CFTR gating modulators. In this study we refer to acute CFTR modulators as gating “activators” (compounds whose administration alone induces CFTR gating) and CFTR “potentiators” (compounds that augment CFTR gating in response to cAMP signaling). These acute modulators include VX-770, a CFTR potentiator shown to restore CFTR activity and improve pulmonary function in G551D-CFTR CF patients (1, 40). These agents may also be applicable to the most common mutation, deletion of phenylalanine at position 508 (F508del), found in 90% of CF individuals. The F508del-CFTR product primarily results in misfolded protein that is subject to elimination during cell processing, resulting in severely reduced expression at the plasma membrane. F508del-CFTR resident at the cell surface also exhibits abnormal cAMP-dependent regulation (5), and defective channel gating (11, 43). Therapeutic approaches intended to correct F508del-CFTR misprocessing by correcting F508del-CFTR misfolding, altering protein chaperones, or addressing other targets (e.g., “correctors”) are under development (25, 40, 41) but are likely to also require rapidly acting modulators to restore F508del-CFTR channel function at the cell surface to clinically relevant levels (41). A better understanding of how acutely acting CFTR modulators promote CFTR activity would allow compounds to be categorized functionally, rationally prioritized for clinical evaluation, and...
may lead to new insights regarding the activation and regulation of CFTR.

In this paper we describe distinct mechanisms of action underlying CFTR activation by acutely active compounds, distinguished by the induction of R-D phosphorylation. We developed a Western blot assay that allows the R-D phosphorylation status to be determined, without radioactivity or obfuscation by CFTR glycosylation status. We found that the isoxazole UC{sub}254 [3-(2-benzyloxy-phenyl)-5-chloromethyl-isoxazole] induced PKA-dependent phosphorylation of the R-D and commensurate activation of wild-type (WT)-, G551D-, and surface localized F508del-CFTR, suggesting that it primarily acts as a CFTR activator. In contrast, the compound VRT-532 [4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)phenol], a pyrazole, primarily augmented cAMP-dependent regulation of CFTR, without evidence of R-D phosphorylation. These data reveal at least two classes among compounds capable of rapidly augmenting CFTR activity. Furthermore, activation of mutant CFTR is possible by a mechanism independent of R-D phosphorylation and is also well suited to restore regulation of CFTR-dependent chloride transport through endogenous (cAMP-dependent) activation pathways, a characteristic shown to be efficacious in the clinical setting (1).

MATERIALS AND METHODS

Ethical approval. The University of Alabama at Birmingham institutional review board approved use of cell lines employed in these studies.

Reagents. CFTR modulators were obtained from the publicly available CFTR modulator library [Dr. Robert J. Bridges, Rosalind Franklin University, Chicago, IL and Cystic Fibrosis Foundation Therapeutics (CFFT), Bethesda, MD] [http://www.cftrfolding.org (July 7, 2009)], and Exclusive Chemistry. All compounds were dissolved at 1,000× in dimethyl sulfoxide (DMSO) and stored at −80°C.

Constructs. The cDNA for human CFTR R-D (residues 635–836) with the addition of a carboxy-terminal hemagglutinin (HA) tag was inserted into pcDNA3.1 (Invitrogen), and the identity of the construct confirmed via DNA sequencing. A retroviral vector was constructed by subcloning the HA-tagged R-D cDNA into pMXpie (provided by Dr. Alice Mui, Jack Bell Research Centre). This vector drives expression of enhanced green fluorescent protein from an internal ribosomal entry site downstream of the R-D cDNA and features a puromycin resistance gene governed by a separate promoter.

Cell lines and cell culture. A-haaggregated CFTR R-D was expressed in HEK293, COS7, or NIH-3T3 cells. BOSC23 virus packaging cells and NIH-3T3 fibroblast cells were cultured in DMEM with penicillin (100 U/ml), streptomycin (50 U/ml), and 10% fetal calf serum. NIH-3T3 cells were retrovirally transduced by incubation with superconcentrates from BOSC23 cells that had been transiently transacted with pMXpie-RD constructs in the presence of 10 μg/ml Polybrene, as previously described (14). Infected cells were selected in puromycin (2 μg/ml).

CFBE41o− cells stably expressing WT- and F508del-CFTR following lentiviral transduction were maintained in DMEM/F12 medium (Invitrogen) with 10 μg/ml fetal calf serum (15). Fischer rat thyroid (FRT) cells stably transduced with WT-, F508del-, or G551D-CFTR cDNA (expression driven by a cytomegalovirus promoter) were a gift from Dr. Luis Galietta (G551D; Gaslini Institute, Genoa, Italy) or Dr. Joseph Zabner (WT and F508del; University of Iowa, Iowa City, IA), and maintained in minimal essential media with penicillin (100 U/ml), streptomycin (50 U/ml), 10% fetal calf serum, 1% l-glutamine, and 25 mM NaHCO3 (with the addition of 600 μg/ml zeocin to maintain G551D selection). Cells were maintained in a 37°C humidified incubator in 5% CO2 except where otherwise specified.

R-D phosphorylation and Western blotting. Cells expressing HA-tagged R-D were treated with potentiatior or vehicle (DMSO) for 20 min unless otherwise specified. Forskolin (20 μM) treatments were for 5 min and H89 (10 μM) pretreatment for 20 min. Potentiator treatments were at the previously published EC{sub}50 (VRT-532, 5 μM; UC{sub}254-152, 50 μM; PG-01, 300 nM; SF-03, 30 nM; UC{sub}253, 3 μM; ΔF508act-02, 70 nM; genistein, 30 μM; UC{sub}209, 2 μM; UC{sub}180, 10 μM) (7, 26, 32, 37, 41, 51), except where otherwise specified. After drug treatment, cells were lysed in buffer (1% NP-40, 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM MgCl2, 15% glycerol) with protease (Complete mini protease cocktail, Roche Diagnostics) and phosphatase inhibitors [NaN{sub}4, 0.2 mM, NaF (10 mM), MoO{sub}4 (1 mM), and β-glycerophosphate (50 mM)]. Equal amounts of total cell lysate were combined with SDS loading buffer for Western blot analysis. For immunoprecipitation studies, 500 μg of total cell lysate was rotated with 20 μl anti-HA agarose affinity matrix (Roche) for 1 h at 4°C, washed with lysis buffer, and combined with SDS loading buffer. Samples were resolved via electrophoresis through a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and visualized by transfer to nitrocellulose and immunoblot with antibody to the HA tag (Covance) or anti-phospho(Ser/Thr) PKA substrate antibody (Cell Signaling, Boston, MA). Phosphorylation of the R-D was visualized as a 2- to 4-kDa shift, as described previously (8).

Ussing chamber analysis of epithelial cell monolayers. FRT and CFBE41o− cells expressing F508del-, G551D-, or WT-CFTR were seeded on fibronectin-coated 0.4-μm permeable supports (Costar, Bethesda, MD; 5 × 10{sup}5 cells/filter, 6.5 mm diameter), grown to confluence, transferred to an air-fluid interface until high (>1,000 Ω/cm²) resistance was achieved, and mounted in modified Ussing chambers (Jim’s Instruments) under voltage-clamp conditions (5, 31). Monolayers were bathed on both sides with Ringer solutions containing 115 mM NaCl, 25 mM NaHCO₃, 2.4 mM KH₂PO₄, 1.24 mM K₂HPO₄, 1.2 mM CaCl₂, 1.2 mM MgCl₂, and 10 mM Na-glucose (pH 7.4). Bath solutions were vigorously stirred and gassed with 95% O₂/5% CO₂, and maintained at 37°C. Short-circuit current (Isc) measurements were obtained by using an epithelial voltage clamp (Univ. of Iowa Bioengineering). A 3-s pulse of 1-s duration was imposed every 100 s to monitor resistance, which was calculated using Ohm’s law. To measure stimulated Isc, the mucosal bathing solution was changed to a low-Cl− solution (1.2 mM NaCl). Sodium gluconate (115 mM) was added, as well as 100 μM amiloride to inhibit sodium transport. Acute modulators (UC{sub}252, 50 μM; VRT-532, 5 μM, forskolin (2 μM), and genistein (50 μM) were added to the bathing solutions as indicated (minimum 5 min of observation at each concentration). Glibenclamide (200 μM) or CFTRinh-172 (10 μM) was added to the mucosal bathing solution at the end of experiments to block CFTR-dependent Isc, as an additional control. To correct F508del-CFTR processing, monolayers were incubated at low temperature (25°C) for 4 h or exposed to Corr-4a (10–20 μM for 8–24 h) (25). At least four monolayers were evaluated per condition.

ELISA cAMP quantification. Cellular cAMP was measured by using an ELISA-based detection kit (Cayman Chemicals) as described previously (5). Briefly, NIH-3T3 cells stably expressing R-D were grown on 35-mm dishes (~2.5 × 10{sup}5 cells/dish), stimulated with agonist for 20 min (UC{sub}252, 50 μM; VRT-532, 5 μM; forskolin, 20 μM), and lysed in 0.1 M HCl, and the cAMP levels were quantified according to the manufacturer’s instructions by the relative standard curve method.

FRET cAMP localization. For ratiometric fluorescence resonance energy transfer (FRET), HEK293 cells expressing a sensor construct that consists of part of the exchange protein activated by cAMP (EPAC) sandwiched between cyan and yellow fluorescent proteins (CFP-EPAC-YFP) were seeded on 35-mm glass-bottom...
dishes and grown for 24–48 h, washed twice with HBSS, and mounted on an inverted Olympus microscope (IX51, U-Plan Fluorite 60 × 1.25 NA oil-immersion objective). Images were recorded by using a cooled EM-CCD camera (Hamamatsu) and the system was controlled by Slidebook 4.2 software (Intelligent Imaging Innovations). The light source used was a 300-W xenon lamp with a neutral density filter. A JP4 CFP/YFP filter set was used for image capture (Chroma), which includes a 430/25-nm excitation filter, a double dichroic beam splitter, and two emission filters (470/30 nm for CFP and 535/30 nm for FRET emission) alternated by filter-change controller Lambda 10–3 (Sutter Instruments, Novato, CA). Time-lapse images were captured with 100- to 300-ms exposure time and 1-min time intervals. After establishing the baseline, forskolin, P1, or P10 were added. Acquired fluorescent images were background subtracted and multiple regions of interest (10–20) were selected (three to five cells) for data analysis by using a ratio module. The emission ratio image (CFP/FRET) was generated at different time points from CFP and FRET emission of background subtracted cells. All calculations were performed by using the FRET module of the SlideBook 4.2 software (Intelligent Imaging). The cell images are presented in pseudocolor to highlight the changes in the ratio of CFP/FRET fluorescence intensity.

**PP2A in vitro protein phosphatase assay.** A colorimetric phosphatase activity assay kit (Millipore) was used to detect PP2A activity according to the manufacturer’s instructions. In brief, purified PP2A was combined with pNPP serine/threonine assay buffer (50 mM Tris-HCl, 100 mM CaCl2, pH 7.0; Millipore) and CFTR modulator. Diluted phosphopeptide (K-R-pT-I-R-R) in serine/threonine assay buffer (250 μM) was added and then incubated for 15 min at 30°C. Malachite Green phosphate detection solution was added for 15 min incubation at 30°C. The relative absorbance was measured at λ = 630 nm. Results are from three independent experiments, each performed in duplicate.

**PDE4D in vitro enzymatic activity assay.** Modulator compound effects on PDE4D hydrolytic activity were measured with the Malachite Green assay by using a commercially available kit (Biomol, Plymouth Meeting, PA). Hydrolytic activity is measured as phosphate release from cAMP. Enzymatically active, recombinant PDE4D protein derived from Escherichia coli (Biomol) was used in the assays. All inhibitors were dissolved in DMSO and then added to the assays at the indicated concentrations (the maximal concentration of DMSO was never more than 10%).

**Statistics.** For densitometry, I0, and enzyme activity studies, descriptive statistics (mean, SD, and SE) and paired and unpaired t-tests were performed by use of GraphPad Prism and Microsoft Excel. Multiple comparisons were made by ANOVA, and Pearson’s test was used to calculate correlation coefficients (SPSS). All statistical tests were two sided and were performed at a 5% significance level (i.e., α = 0.05). Error bars represent SE in all figures unless otherwise noted.

**RESULTS**

**R-D phosphorylation status after treatment with CFTR modulator.** Compounds acutely augmenting CFTR activity may act by facilitating phosphorylation of the R-D. The assessment of such phosphorylation is limited by the large size and broad glycosylation-sensitive electrophoresis pattern of full-length CFTR, which interferes with the interpretation of mobility shift experiments. To more accurately measure phosphorylation-dependent events, we developed a phospho-assay system by which HA-tagged R-D of CFTR is expressed heterologously and tested in NIH-3T3 cells. To validate this assay, we analyzed the phosphorylation of the domain by forskolin, which is known to induce PKA-mediated phosphorylation of the CFTR R-D. Treatment of the cells with 20 μM forskolin for 5 min resulted in a 2- to 4-kDa mobility shift of the R-D on Western blots (Fig. 1A) as described previously for isolated R-D (confirmed by γ32-ATP phosphorylation and functional analysis) (8, 22, 50) as well as for CFTR half-channel constructs that included the R-D (23). Forskolin induced the production of high levels of cAMP (Fig. 1F), and the phosphorylation of the R-D by forskolin was inhibited by pretreatment with the PKA inhibitor H89 (10 μM for 20 min) (Fig. 1, A, D, E). Inhibition of PKA at 10 μM (the in vivo IC50) would be predicted to be incomplete but circumvent the nonspecificity exhibited by H89 (the only cell-permeable PKA inhibitor) at higher concentrations (12, 20). The lower mobility band was confirmed to correspond with the phosphorylated R-D band using immunoprecipitation with phospho-Ser/Thr site-specific antibodies (Fig. 1C).

We used this system to compare phosphorylation induced by nine acute CFTR modulators available through the CFFT Modulator Library, compared with the well-characterized CFTR modulator genistein as a control. Treatment of the cells with UC-CF-152 (P < 0.005, n = 7) or UC-CF-029 (P < 0.05) resulted in enhanced R-D phosphorylation compared with the vehicle control (Fig. 1B), with UC-CF-152 levels of phosphorylation ~37% of that stimulated by forskolin and greater than phosphorylation levels stimulated by genistein. Several compounds (UC-CF-853, UC-CF-180, PG-01, ΔF508act-02, and SF-03) elicited higher levels of R-D phosphorylation than vehicle, but these effects were not statistically significant (Tukey’s multiple-comparison test). Notably, treatment with VRT-532 (5 μM × 20 min) did not elicit any detectable phosphorylation of R-D in this assay (Fig. 1, A–D) and was significantly less than UC-CF-152 (P < 0.05). Further analysis of the UC-CF-152 induced phosphorylation of the R-D confirmed that it occurred rapidly (within 2 min of compound exposure, Fig. 1E). Furthermore, pretreatment with H89 reduced the UC-CF-152 induced R-D phosphorylation (Fig. 1D). This suggested that, like forskolin, UC-CF-152 stimulated phosphorylation of the R-D is mediated in part through PKA-mediated phosphorylation event. However, unlike forskolin, UC-CF-152 did not act to stimulate the production of detectable global or perimembranous cAMP in these cells (Fig. 1F and Supplemental Fig. S1, respectively; the online version of this article contains supplemental data), and did not directly activate human PKA (Supplemental Fig. S2). R-D phosphorylation by these compounds was not due to changes in protein phosphatase 2A (PP2A) activity or PDE inhibition. Several protein phosphatases have been implicated in CFTR regulation, and PP2A in particular is thought to be relevant to the CFTR microdomain (38, 42). When PP2A activity was measured via colorimetric reading of phosphate cleavage from a PP2A-specific peptide, neither VRT-532 nor UC-CF-152 altered PP2A activity compared with the positive control inhibitor (20 μM cantharidin and 20 nM microcystin LR) (P < 0.0001; Supplemental Fig. S3). Similarly, inhibition of PDE4D was not observed with either agent (Supplemental Fig. S4). Finally, we determined that VRT-532 did not shift the dose-response of R-D phosphorylation by forskolin, demonstrating that VRT-532 did not potentiate R-D phosphorylation, whereas UC-CF-152 increased phosphorylation in the presence of all concentrations of forskolin (Supplemental Fig. S5).
Fig. 1. Impact of acute cystic fibrosis transmembrane conductance regulator (CFTR) modulators on regulatory domain (R-D) phosphorylation.

A: recombinant R-D was stably expressed in NIH-3T3 cells and detected by Western blotting against the hemagglutinin (HA) tag. Phosphorylated R-D (~25 kDa) is visible after 5-min treatment with forskolin (20 μM, positive control) as a 2- to 4-kDa shift in gel mobility (△ vs. ▲). Forskolin-mediated R-D phosphorylation could be partially inhibited by preincubation with the PKA inhibitor H89 (10 μM). Vehicle alone (DMSO) had no effect. Phosphorylation was seen after incubation with UCcr-152 (50 μM); minimal phosphorylation was seen with VRT-532 (5 μM). Other compounds had intermediate effects. Results are representative of 7 independent experiments.

B: summary data for the compounds are shown, as determined by densitometry. Quantification reflects gel shift from R-D phosphorylation induced by potentiators, normalized to forskolin within the same experiment (*P < 0.05 and **P < 0.005 vs. vehicle, n = 7). Inset: chemical structures of compounds VRT-532 [4-methyl-2-(5-phenyl-1H-pyrazol-3-y)phenol] and UCcr-152 [3-(2-benzyl oxy-phenyl)-5-chloromethyl-isoxazole].

C: forskolin and UCcr-152 induce phosphorylation of R-D at PKA consensus sites. Recombinant R-D was stably expressed in NIH-3T3 cells and detected by Western blotting against the phosphorylated Ser/Thr PKA consensus sites (top) or the HA-tagged R-D (middle and bottom). Middle and bottom are different exposures of the same blot. Preenrichment for the isolated R-D by immunoprecipitation (i.p.) of the HA tag shows binding of the PKA consensus site antibody to the R-D alone (right) after forskolin or UCcr-152 treatment.

D: phosphorylation of R-D by UCcr-152 is reduced by preincubation with H89 for 20 min. Western blotting was performed in triplicate for verification.

E: time dependence studies with UCcr-152. Results shown are representative of at least 3 independent experiments.

F: compounds UCcr-152 (50 μM) and VRT-532 (5 μM) do not elevate cellular cAMP (*P < 0.001, n = 3). Total cellular levels of cAMP with vehicle, UCcr-152, and VRT-532 were below the limits of detection. Forskolin (positive control, 20 μM) increased cAMP.
Impact of UCCF-152 and VRT-532 on CFTR-dependent short-circuit current. Because these two compounds demonstrated different effects on R-D phosphorylation, we next compared the functional activity of UCCF-152 and VRT-532 by using the I_{sc} studies in polarized epithelial monolayers. We first employed the CFBE410- cell line, an SV-40 transformed CF bronchial epithelial cell line that has undetectably low levels of expression of endogenous F508del-CFTR. By using this cell line, I_{sc} can be compared with stable heterologous expression of WT-CFTR, F508del-CFTR, or other mutant forms of CFTR (5, 15). We compared the effects of the UCCF-152 and VRT-532 alone and after sequential addition of submaximal forskolin (2 μM), which activates CFTR through R-D phosphorylation, and genistein (50 μM), which acts primarily on the gating mechanism of surface-expressed CFTR. Submaximal doses of the cAMP agonist forskolin were selected to allow additional detectable activation with other compounds and better discrimination of divergent compound mechanisms. Representative tracings are shown in Fig. 2A, and the summary data are shown in Fig. 2, B–E.

WT-CFTR. Although addition of either VRT-532 or UCCF-152 resulted in an I_{sc} increase greater than vehicle control (Fig. 2A, left, and B), UCCF-152 induced activity was significantly greater than that induced by VRT-532 (P < 0.05) and approached the response induced by forskolin alone (Fig. 2C, vehicle pretreatment). Prior addition of VRT-532 enhanced I_{sc} induced by subsequent addition of forskolin addition (e.g., “potentiated” cAMP activation of the channel) (Fig. 2C). In contrast, cells pretreated with UCCF-152 showed minimal additional I_{sc} with forskolin (Fig. 2C). Subsequent addition of genistein did not result in a further increase in the UCCF-152 or VRT-532-treated CFBE/WT-CFTR cells (Fig. 2D). These findings suggest that UCCF-152 primarily activated CFTR in a fashion redundant with forskolin, whereas VRT-532 potentiated forskolin mediated activation and only modestly activated CFTR alone.

In biochemical studies both UCCF-152 treatment and forskolin, but not VRT-532, stimulated the phosphorylation of CFTR R-D, an effect reduced by H89. We therefore investigated the effects of H89 pretreatment on the I_{sc} of CFBE/WT-CFTR cells treated with CFTR modulators (Fig. 3). Pretreatment with H89 resulted in a significant reduction (71%, P < 0.05 for UCCF-152 vs. vehicle) in UCCF-152-stimulated I_{sc}. In contrast, H89 reduced VRT-532-induced I_{sc} by only 35% (P = not significant for VRT-532 vs. vehicle). These results indicate that the mechanism by which UCCF-152 enhances CFTR activity overlaps with the mechanism of action of forskolin and is largely dependent on PKA phosphorylation. They also suggest that the mechanism by which VRT-532 enhances CFTR activity differs substantially from the mechanism underlying UCCF-152 and forskolin.

F508del-CFTR. To test whether differences between UCCF-152 and VRT-532 were also observed in clinically relevant CFTR mutants, we next examined the agents’ impact on F508del-CFTR activity following temperature rescue to the cell surface by preincubation at 27°C (R-F508del-CFTR). Forskolin treatment of temperature-rescued CFBE410- cells (CFBE/R-F508del-CFTR) indicated that these cells were relatively resistant to cAMP stimuli (Fig. 2, A, right, and B–E), whereas genistein robustly enhanced I_{sc} as previously described (5), indicative of the gating defect present in R-F508del-CFTR. Both VRT-532 (13.5 μA/cm²) and UCCF-152 (7.3 μA/cm²) increased I_{sc} in the CFBE/R-F508del-CFTR (Fig. 2B). The compounds in combination elicited a synergistic response, further suggesting divergent mechanisms. Notably, the responses to subsequent addition of forskolin differed substantially. The addition of forskolin did not enhance I_{sc} in the UCCF-152-treated cells, regardless of the presence or absence of concurrent VRT-532, whereas I_{sc} was strongly augmented by forskolin in cells pretreated with VRT-532 alone (Fig. 2C). In addition, total stimulated I_{sc} (modulator + forskolin + genistein) in UCCF-152-treated cells was 42.2 μA/cm², 48.5 μA/cm² for cells pretreated with VRT-532 and UCCF-152 simultaneously, and 39.4 μA/cm² in vehicle (vehicle + forskolin + genistein), whereas total stimulated I_{sc} for VRT-532-pretreated cells was 49.4 μA/cm² (P < 0.05 vs. vehicle pretreated) (Fig. 2E). These results indicated that VRT-532, but not UCCF-152, potentiated I_{sc} activation by forskolin in the F508del-CFTR-expressing cells, overcoming the CFBE/ R-F508del-CFTR defect in cAMP-dependent activation seen in vehicle-treated cells.

Some elements of CFTR activation have been found to be dependent on cell type. FRT cells have been used in several high-throughput screening programs and studies to compare modulator effects (17). Expanding on results seen in the CFBE410- model, we evaluated the impact of modulator compounds on stably expressed CFTR in FRT cells grown in polarizing conditions. In this setting we examined two clinically relevant CFTR mutations: R-F508del-CFTR and G551D-CFTR. In FRT/R-F508del cells (Fig. 4, left), UCCF-152 again activated I_{sc} (Fig. 4A) but had no effect on forskolin-dependent I_{sc} (Fig. 4B). In contrast, VRT-532 had minimal detectable effect on I_{sc} upon direct addition (Fig. 4A) but strongly potentiated forskolin-dependent I_{sc} (Fig. 4B). Similarly, total current (modulator + forskolin) was augmented by VRT-532, and less so by UCCF-152 treatment (Fig. 4C).

G551D-CFTR. We then tested the effects of modulators on G551D-CFTR, a surface mutant of CFTR that is associated with a severe gating defect (Fig. 4, right) (49). Again UCCF-152 enhanced I_{sc} upon acute addition (Fig. 4A) but did not potentiate cAMP-dependent CFTR activation (Fig. 4B). Potentiation of forskolin was observed in the FRT/G551D-CFTR cells treated with VRT-532 (Fig. 4B), reflecting the propensity of VRT-532, but not UCCF-152, to rescue cAMP-induced I_{sc} in the setting of a CFTR gating mutation. Both agents significantly increased total I_{sc}, although VRT-532-treated cells had greater total activated current (Fig. 4C; 5.5 ± 0.7 μA/cm² VRT-532 vs. 3.7 ± 0.8 μA/cm² UCCF-152, P < 0.05).

Modulators in combination with F508del-CFTR correctors. To investigate the combined use of small molecule correctors of F508del-CFTR with rapidly acting CFTR modulators, as proposed for clinical testing, we compared the effects of the compounds on cells in which the F508del-CFTR had been rescued to the cell surface by treatment with the small molecule corrector of misprocessing Corr-4a (Fig. 5) (25). Corr-4a alone did not overcome the block of forskolin-mediated activation of CFBE/F508del-CFTR cells that is characteristic of these cells, although the agent did increase I_{sc} stimulated by the combination of genistein and forskolin (7.5 ± 1.1 μA/cm² 37°C control, 11.0 ± 1.2 μA/cm² Corr-4a treated, P < 0.01, n = 14 per condition). Similar to the results obtained in the setting of low-temperature rescued CFTR, VRT-532 significantly en-
Fig. 2. Activation of CFTR-dependent short-circuit current (\(I_{sc}\)) in polarized epithelial monolayers by UCCF-152 and VRT-532. \(I_{sc}\) in CFBE41o \(^{-}\) monolayers stably expressing WT- or F508del-CFTR was measured in modified Ussing chambers. A: after establishment of a serosal to mucosal Cl\(^{-}\) gradient and addition of amiloride (100 \(\mu\)M, LC/Amil), monolayers were sequentially exposed to test compound (UCCF-152, 50 \(\mu\)M; VRT-532, 5 \(\mu\)M; UCCF-152, 50 \(\mu\)M / VRT-532, 5 \(\mu\)M) or vehicle (V), forskolin (2 \(\mu\)M, F), genistein (50 \(\mu\)M, G), and glibenclamide (200 \(\mu\)M, Glb). Representative tracings demonstrating VRT-532, UCCF-152, or vehicle stimulation of cells expressing WT-CFTR or F508del-CFTR are shown. B–E: summary data show the stimulated increase in \(I_{sc}\) for WT-CFTR (open bars) and F508del-CFTR (light shaded bars) following sequential addition of modulator (B), forskolin (C), genistein (D), and total stimulated \(I_{sc}\) (E). *\(P < 0.05\), **\(P < 0.01\), and ***\(P = 0.06\) compared with vehicle in each respective cell line. Results shown are representative of a minimum of 2 experiments, with total of \(n = 4\) (WT) and 16 (F508del) monolayers.
hanced forskolin-mediated activation in cells in Corr-4a-treated cells whereas UC-CF-152 pretreatment had a minimal effect on forskolin-stimulated Cl\textsuperscript{-} transport (Fig. 5A). VRT-532 also significantly enhanced forskolin-mediated activation in both CFBE410- and FRT cells in which F508del had not been rescued to the cell surface by low-temperature growth (37°C control), likely reflecting low levels of surface localized F508del-CFTR under basal conditions in these cells.

Effect of VX-770 on R-D phosphorylation. Recent clinical testing has demonstrated that the CFTR potentiator VX-770 shows significant promise for the treatment of G551D-CFTR, providing partial restoration of chloride conductance across both nasal epithelia and sweat glands in human subjects (1). Although the precise mechanism of action remains to be defined (30), VX-770 strongly potentiates forskolin-dependent current in G551D-CFTR monolayers (40). Assessment of this CFTR gating potentiator demonstrated minimal phosphorylation of the R-D (Fig. 6), similar to that seen with VRT-532. These data indicate that, like VRT-532, VX-770 potentiates CFTR activity without direct impact on R-D phosphorylation.

DISCUSSION

Here we describe two distinct mechanisms underlying acute CFTR activation by CFTR modulators recently identified in high-throughput screening programs. The compound UC-CF-152 (an isoxazole) induced robust phosphorylation of the R-D and increased I\textsubscript{sc} in WT, R-F508del-, and G551D-CFTR cell monolayers in a fashion that was relatively sensitive to PKA inhibition but did not increase (and in some cases reduced) subsequent cAMP-mediated activation of CFTR by forskolin. In contrast, VRT-532 (a pyrazole) did not induce phosphorylation of the R-D and activated CFTR through a mechanism that was relatively independent of H89 blockade. Potentiation of downstream forskolin-dependent activation was observed following addition of VRT-532 in F508del-, G551D-, and WT-CFTR cell monolayers. These data indicate that UC-CF-152 is most appropriately classified as an activator of Cl\textsuperscript{-} conductance, increasing I\textsubscript{sc} regardless of endogenous cAMP-dependent signaling, whereas VRT-532 primarily potentiates cAMP-mediated activation. Consistent with this characterization, the two agents exhibit distinctly different effects on R-D phosphorylation status and synergistically increase I\textsubscript{sc} in R-F508del-CFTR monolayers, indicating important functional implications of the mechanistic divergence at the crucial R-D phosphorylation step in CFTR activation.

The present studies demonstrate that UC-CF-152 induces potent and rapid phosphorylation of the R-D. The specific UC-CF-152 target(s) responsible for this modification is not yet known. However, the rapid time course, combined with recent evidence regarding the kinetics of individual R-D phosphorylation sites (27), may allow future speculation of the specific sites relevant to UC-CF-152 activity. Further work to identify the phosphorylated residues involved, combined with recent efforts to elucidate the impact of individual phosphorylation sites on CFTR conformational changes during gating, may expand our understanding of both CFTR gating and the impact of UC-CF-152. For example, the phosphorylation sites within the R-D known to be relevant to endogenous CFTR activation, some (including S768) are thought to be inhibitory and may address the impact of UC-CF-152 on total activation in some cell types (e.g., WT-CFTR CFBE410\textsuperscript{-}). Additionally, one phosphorylation site, S422, lies outside the region encompassed by the R-D assay, in a portion of CFTR known as the regulatory insertion of NBD1. As shown by Sammelson et al. (32) and confirmed by our study, UC-CF-152 did not increase levels of cellular cAMP, nor did it directly activate PKA, indicating a mechanism distinct from CFTR activation by adenylyl cyclase. Similarly, neither VRT-532 nor UC-CF-152 inhibited PP2A or PDE4 in a cell-free system. Evidence suggests these are the most relevant protein phosphatases and phosphodiesterases to CFTR microenvironment regulation, but other isoforms may be considered in further work. Enhancement of R-D susceptibility to phosphorylation, or activation of PKC (34) (an alternative CFTR phosphorylation pathway only partially susceptible to H89 blockade) are potential pathways through which CFTR may be phosphorylated by UC-CF-152. Alternatively, because phosphodiesterases of the PDE4 family are thought to be compartmentally active in epithelia (4), measurements of cAMP in closer proximity to CFTR may be required to discern a local effect. Experiments to examine whether blockade of these candidate pathways also inhibit R-D phosphorylation by UC-CF-152 will be necessary to distinguish among these possibilities.

Wang et al. (43) demonstrated that F508del-CFTR may be a poorer substrate for PKA phosphorylation than WT-CFTR, since CFTR activity in an isolated cell-membrane patch in the presence of low levels of PKA exhibits gating behavior similar to F508del-CFTR. Our data suggest that normal regulation of the mutant channel can be restored by a compound capable of pharmacologically potentiating endogenous signaling and does not require phosphorylation by the compound itself. It should be noted that VRT-532 or VX-770 may induce phosphorylation events below the resolution of the R-D assay, although such events would be expected to be evident with application of the phosphorylation-specific antibody. Moreover, these observations suggest for the first time that a potentiator (VRT-532) can rescue cAMP-dependent activation in CFBE410\textsuperscript{-} cells, an airway cell type representative of most primary F508del bronchial epithelial cells, in that it is refractory to cAMP-mediated stimulation of R-F508delCFTR through physiological receptor-mediated pathways (5). These findings are germane and contribute to previous reports of the activity of...
VRT-532 and VX-770 in primary epithelial cells (41). Furthermore, the observation that augmentation of the forskolin-stimulated Cl⁻/H⁺ current in uncorrected F508del-CFTR expressing CFBE41o/H₁₁₀₀² cells was detectable following application of VRT-532 [an effect not elicited by flavonoid CFTR modulators (5)], provides evidence that low levels of F508del-CFTR at the cell surface can be detected in this model system, as has also been suggested in some human studies of primary human cells (13, 35, 41).

Our findings suggest that the mechanism of action of VRT-532 is downstream of R-D phosphorylation. CFTR active compounds have been previously postulated to interact directly with the NBDs, including the well-studied flavonoid genistein (44). In this study, unlike VRT-532, genistein induced highly variable levels of R-D phosphorylation (Fig. 1A) and, in both this study (Fig. 2) and others, failed to restore cAMP response in the CFBE41o⁻ and CFBE/R-F508delCFTR systems. Van Goor et al. (41) postulated a direct interaction between VRT-532 and CFTR based on single-channel and cell signaling analyses, a notion supported by the dual effects of this compound as a CFTR potentiator and corrector of F508del-CFTR maturation and processing (45). Wang et al. (45) used chemical cross-linking to demonstrate that VRT-532 induced a change in orientation of the transmembrane domains, thus inferring a direct CFTR/VRT-532 interaction. The conformation of the transmembrane domains is affected by interactions with other CFTR domains, particularly the NBDs (21, 36, 46, 47). Wellhauser et al. (48) and Pasyk et al. (24) confirmed a direct interaction between VRT-532 and the CFTR protein, also in a manner that supported F508del- and G551D-CFTR gating. Several groups have provided evidence that R-D phosphorylation and NBD1-NBD2 dimerization are interdependent (3, 8, 13, 21, 36, 37, 48).

**Fig. 4.** VRT-532, but not UCCF-152, potentiates forskolin in 2 different gating mutations. Iₘ in Fischer rat thyroid (FRT) monolayers stably expressing F508del- (light shaded bars) or G551D-CFTR (dark shaded bars) was measured in modified Ussing Chambers. After establishment of a serosal-to-mucosal Cl⁻ gradient and addition of amiloride (100 μM), monolayers were sequentially exposed to modulator (UCCF-152, 50 μM; VRT-532, 2 μM) or vehicle, forskolin (2 μM), and CFTRINH-172 (10 μM). Data shows the increase in Iₘ following sequential addition of each modulator (A), forskolin (B), and the total stimulated Iₘ. UCCF-152 directly stimulated G551D-CFTR and F508del-CFTR Iₘ (*P < 0.05 compared with vehicle). Subsequent activation of G551D- and F508del-CFTR by forskolin was significantly increased by pretreatment with VRT-532 (**P < 0.01 compared with vehicle pretreatment). Results shown are representative of a minimum of 2 experiments, n = 4 monolayers per experiment.
and that proper NBD conformation can have an impact on channel sensitivity to R-D activation by PKA (5, 43). For example, deletion of phenylalanine 508 has been shown by macropatch analysis to attenuate phosphorylation-dependent activation of CFTR (43), a finding confirmed in polarized epithelial cells (5). It is plausible that CFTR interactions with VRT-532 may increase the sensitivity of CFTR to both endogenous and agonist-stimulated cAMP signaling, effectively resolving the defective cAMP signaling response seen in F508del-CFTR expressing cells (Fig. 2). That VRT-532 did not shift the forskolin dose-response of the R-D assay indicates that these interactions between VRT-532 and CFTR, whether direct or indirect, are outside of the R-D.

It bears noting that the mechanistic basis of each compound appears to be closely related to the HTS method through which they were identified. VRT-532 was identified by use of a HTS system that measured activity in F508del-CFTR following application of both potentiator and forskolin (10 μM) whereas UC\textsubscript{CF}-152 and UC\textsubscript{CF}-029 (which also increased R-D phosphorylation) were identified in WT cells in the absence of addition of forskolin (41). This explains why VRT-532 increases CFTR activation of CFTR (43), a finding confirmed in polarized epithelial cell monolayers. The results of our studies indicate that combined use of an F508del-CFTR processing corrector and CFTR potentiator of endogenous and agonist-stimulated cAMP signaling, effectively resolving CFTR defects. VRT-532 retains its property as a CFTR potentiator in the setting of processing restoration by either low temperature or the small molecule corrector, Corr-4a. Whereas some primary F508del bronchial epithelial cells exhibit intact cAMP-dependent activation [likely due to cell-type specific aspects (6)], previous studies in our laboratory (5) and others (2, 43) provide evidence that a F508del-CFTR potentiator will likely be needed to produce clinically relevant levels of CFTR activity in the airways of subjects harboring F508del, unless the pharmacological approach employed to correct the processing defect also inherently confers wild-type gating of the mutant channel in sufficient numbers.

An improved understanding of the mechanisms of action of newly described acute CFTR modulators is essential for the design of congeners, the identification of agents best suited for use in combination with correctors of F508del-CFTR processing, or for application to particular CF mutations. The results of our studies suggest that there are at least two classes of acute CFTR modulators characterized by the effects on R-D phosphorylation and that these results distinguished activation (as opposed to potentiation) of CFTR-mediated currents and predicted potential for synergy between agents. Results reported for the CFTR potentiator VX-770 on cells (40) and in CF patients carrying the G551D-CFTR mutation (1) combined with our studies indicate that augmentation of R-D phosphorylation is not required to confer rescue of mutant CFTR channel activity in the clinic. Analysis of R-D phosphorylation may be useful to categorize acute CFTR modulators and help distinguish the mechanistic basis underlying their effects.

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DISCLOSURES

S. M. Rowe served on the advisory board for Vertex Pharmaceuticals, Inspire Pharmaceuticals, and PTC Therapeutics, Inc.
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