Pharmacological modulation of cGMP levels by phosphodiesterase 5 inhibitors as a therapeutic strategy for treatment of respiratory pathology in cystic fibrosis

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1Department of Molecular Genetics and Microbiology, 2College of Pharmacy, Departments of 3Internal Medicine, 4Pediatrics, and 5Cell Biology and Physiology, University of New Mexico Health Science Center, Albuquerque, New Mexico; 6Department of Biochemistry and Microbiology, Joan C. Edwards School of Medicine Marshall University, Huntington, West Virginia; and 7Department of Biochemistry, Erasmus University Medical Center, Rotterdam, The Netherlands

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Pharmacological modulation of cGMP levels by phosphodiesterase 5 inhibitors as a therapeutic strategy for treatment of respiratory pathology in cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 293: L712–L719, 2007. First published June 22, 2007; doi:10.1152/ajplung.00314.2006.—The CFTR gene encodes a chloride channel with pleiotropic effects on cell physiology and metabolism. Here, we show that increasing cGMP levels to inhibit epithelial Na+ channel in cystic fibrosis (CF) respiratory epithelial cells corrects several aspects of the downstream pathology in CF. Cell culture models, using a range of CF cell lines and primary cells, showed that complementary pharmacological approaches to increasing intracellular cGMP, by elevating guanylyl cyclase activity though reduced nitric oxide, addition of cell-permeable cGMP analogs, or inhibition of phosphodiesterase 5 corrected multiple aspects of the CF pathological cascade. These included correction of defective protein glycosylation, bacterial adherence, and proinflammatory responses. Furthermore, pharmacological inhibition of phosphodiesterase 5 in tissues ex vivo or in animal models improved transepithelial currents across nasal mucosae from transgenic F508del Cftrtm1Eur mice and reduced neutrophil infiltration on bacterial aerosol challenge in Pseudomonas aeruginosa-susceptible DBA2 mice. Our findings define phosphodiesterase 5 as a specific target for correcting a number of previously disconnected defects in the CF respiratory tract, now linked through this study. Our study suggests that phosphodiesterase 5 inhibition provides an opportunity for simultaneous and concerted correction of seemingly disparate complications in CF.

Cystic fibrosis (CF) is the most common life-shortening inheritable disease in Caucasians, caused by mutations in the gene encoding CF transmembrane conductance regulator (CFTR) (41). CFTR, apart from being a chloride channel (1), confers pleiotropic effects on other cellular functions (26, 37). The synergisms between CFTR and other systems have led to a search for organ-specific modifier genes and factors, since the CFTR genotype alone does not always correlate with severity of respiratory disease (10, 14).

Recent studies (31) unequivocally point to the pivotal role of a runaway sodium transport in CF lung pathology, secondary to the loss of epithelial sodium channel (ENaC) regulation in CFTR-mutant respiratory epithelial cells (46). This defect is responsible for respiratory tract problems, including airway surface liquid hyperabsorption (31) and hyperacidification of the trans-Golgi network (TGN) in CF respiratory epithelial cells (38), although the mechanism and signaling events linking upstream defects in CF and TGN hyperacidification remain to be delineated. The previously reported normally acidic TGN lumen in CF cells causes suboptimal sialyltransferase activity (38), resulting in defective sialylation of plasma membrane glucocerebrosides providing neoreceptors for bacterial adherence, thus promoting chronic bacterial colonization and inflammation in the CF lung (3, 7, 22, 38). We have recently reported (39) our initial observations that hyperacidification of another intracellular compartment in CF cells, i.e., recycling endosome, is affected by cGMP levels and a phosphodiesterase activity, but the exact signaling pathway and specific components involved have not been identified.

Here, we present siRNA knockdown-based experiments demonstrating directly that ENaC activity is responsible for organellar hyperacidification in CF cells and show that phosphodiesterase 5 (PDE5) can be specifically targeted to normalize lumenal pH in the TGN of CF respiratory epithelial cells. We demonstrate the causal relationships in CF cells (see model in Fig. 1), starting with their reduced nitric oxide levels, leading to lower than normal cGMP levels and ending with the effects on hyperacidification in CF based on unrepressed ENaC and reduced PDE5 levels. We furthermore show that the PDE5 inhibitor sildenafil corrects the majority of the known pathological defects in CF by reducing bacterial adherence to respiratory epithelial cells and normalizing the overexuberant proinflammatory response to bacterial products. Inhibition of PDE5 provides beneficial effects in ex vivo studies with respiratory mucosal tissue from transgenic mice and in an aerosol infection model with mice susceptible to Pseudomonas aeruginosa, the principal bacterial pathogen in CF.
PHARMACOLOGICAL MODULATION OF cGMP LEVELS IN CF

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Verkman. 5-Cyclopropyl-2-[1-(2-fluoro-benzyl)]-1H-pyrazolo[3,4-b]pyridine-3-ylpyrimidin-4-ylamine (BAY-41-2272), an NO-independent activator of NO-sensitive guanylyl cyclase (5), was from Sigma.

Cells and tissue culture. IB3–1 is a bronchial epithelial cell line derived from a CF patient with a DF508/W1282X CFTR mutant genotype (55). S9 is a derivative of IB3–1 corrected for chloride conductance by stable transfection with a functional CFTR. The cells were maintained in LHC-8 media (Biosource). The CF cell line CFBE41o– (DF508/DF508) and normal human bronchial epithelial cells16HBEo– (from D. Gruenert) were maintained in complete MEM-media (Gibco, Life Technologies). The normal human tracheal cell line 9HTEo–, transfected with pCep-R or pCep, were from P. Davis and were maintained in complete DMEM (Gibco, Life Technologies). Primary bronchial epithelial cells were from Clonetics (NHBE) or from P. Karp (CF lung transplants) and were maintained in supplemented BEBM (Cambrex, MD). CFTR genotyping was carried out by PCR and oligonucleotide ligation assay. Primary cells and multiple cell lines, as genetically or otherwise matched pairs (CF and normal phenotypes), when available, were used. In this study the results represent nearly all published lines available and developed for CF research by independent groups, with the exceptions of 1) CFT1 cells (36), since their culture requires chola toxin added to the medium and consequently were not included in this study; and 2) CuFi cell lines (54), since these cells could not be used in our Pseudomonas adhesion assay since the monolayers were highly sensitive to addition of bacteria.

Transfections and microscopy. TGN38 fusion with pH-sensitive GFP (33) was from J. Rothman. Cells were transfected with 1 µg/ml DNA using Effectene (Qiagen) for 48 h. Ratiometric pH determination by fluorescence microscopy in live cells was carried out as previously described (33, 38, 40). A pH standard curve was generated by collapsing the pH-gradient by incubating cells in 10 mM monensin and 10 mM nigericin for 30 min at 37°C in buffer A at pH 7.4, 6.5, or 5.5, and ratios were recorded for internal standards. Fluorescence images were taken on excitation at 410 and 470 nm (6 consecutive exposures). Three regions of interest were selected, and the standard curve was plotted as averaged 410:470 ratio values for a given buffer and pH. The term hyperacidification for TGN in CF cells, is used relative to the less acidic pH in normal or CFTR-corrected cells. It is known that the pH of TGN is elevated in airway epithelial cells compared with a number of other cell types (49). Confocal fluorescence microscopy of fixed samples was carried out on Zeiss 510 META microscope. Endogenous ENaC was revealed by immunoblots or immunofluorescence (ENaCα antibody from Diagnostic).

Manipulation of signaling pathways affecting pH of TGN in CF cells. All compounds were diluted in buffer A before addition. NO donors DETA-NONOate or NORM were added for 20 min at a concentration that initially released 0.1 mM NO under physiological conditions. Nitric oxide synthase was inhibited in S9 cells by incubation with 1 mM l-NAME or R-NAME for 60 min. Ten millimolar guanylin (28) or protoporphyrin IX (21) (guanylate cyclase activators) were added for 15 and 90 min, respectively. Ten micromolar ODQ (51) or NS-2028 (8) (guanylate cyclase inhibitors) were added for 30 min followed by incubation of the cells with DETA-NONOate, as described above. One hundred micromolar 8-Br-cGMP (51) or dibutyryl-cGMP (51) was added for 60 or 30 min, respectively. Phosphodiesterase inhibitors, 200 µM IBMX (23), 100 nM MBCQ, 10 µM rolipram, 1 or 50 µM zaprinast, and 300 nM sildenafil, were added for 60 min at 37°C. Ten micromolar acetylstrophatedine was added for 1 h before addition of 300 nM sildenafil. S9 cells were incubated with 0.6 µM CFTRinh-172 for 15 min followed by three 10-min washes with buffer A. To block intracellular ENaC, cells were treated for 60 min with 10 µM benzamil, a membrane permeate derivative of the sodium channel blocker amiloride.

ENaCα and PDE5 knockdown with siRNA. All siRNAs were from Dharmacon and generated using specific algorithms for exclusive

METHODS

Chemicals. Sildenafil (2) was prepared by purification as previously described (16) and used at therapeutically relevant concentrations of 300 nM (http://www.viagrand.com) with pharmacokinetics not affected by azithromycin (37), a drug frequently prescribed in CF. The following compounds were from BIOMOL Research Laboratories: N-[3-(aminomethyl)benzyl]acetamidine (1400W), a highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo (18), N-nitro-l-arginine methyl ester (l-NAME), N-nitro-l-arginine methyl ester (R-NAME), (±)-3-(E)-ethyl-2’-((E)-hydroxyimino-5-nitro-3-hexene-carboxamido) pyridine (NOR-4), (Z)-1-[2-(aminomethyl)N-2-ammonioethy]laminol]azaien-1-ium-1,2-diolate (DETA-NONOate), 2-(4-carboxyphenyl)4,4,5,5 tetramethyl-imidazoline-1-oxyl-3-oxide (CPTIO), guanylin, protoporphyrin IX, 1H-oxadiazolo[4,3-a]-quinoxalin-1-one (ODQ), 4H-8-bromo-1,2,4-oxadiazolo[3,4-d]benz(b)- (1,4)oxazin-1-one (NS-2828), 3-isobutyl-1-methylxanthine (IBMX), (±)-4-[3-(cyclopropylxoxy)-4-methoxophenyl]-2-pyrrolidinone (rolipram), 4-[3-(4-methyl-1-ethylxan)ilybenzyl]aminol]-6-chloro-4-azidocyanoline (MBCQ) (17), 2-(2-propanoylphenyl)-8-azahyoxanthine (Zaprinast), 8-Br-cGMP, and dibutyryl-cGMP. N-(benzylamidino)-3,5-diamino-6-chloropyrazine-carboxamide (benzamil) was from Alexis. The specific CFTR chloride channel inhibitor CFTRinh-172 (30) was from A.

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For more detailed discussion, see supplementary data available online at the American Journal of Physiology–Lung Cellular and Molecular Physiology website.

Fig. 1. Model of defects in cystic fibrosis (CF) lung epithelial cells. A and B are based on a previously published model (37). A: in normal cells, a functional CFTR inhibits epithelial sodium channel (ENaC). As a result, positive charge from Na+ (bold), pumped into the lumen by Na+-K+ATPase, builds up membrane potential, which shuts down H+ dependent inhibition of ENaC. This results in Na+ or inhibition of cGMP-specific phosphodiesterase PDE5, results in a cGMP-ficiation of the organellar lumen. C: addition of exogenous nitric oxide (NO•) or inhibition of cGMP-specific phosphodiesterase PDE5, results in a cGMP-dependent inhibition of ENaC. This results in Na+ build-up (bold) in the correction of organellar pH, even in the absence of CFTR in CF cells, normalizing organellar acidification. When Na+-K+ATPase is inhibited by acetylstrophatedine, reduced amounts of Na+ are pumped into the lumen (as indicated by parentheses), which causes hyperacidification in normal or CFTR-corrected cells. For more detailed discussion, see supplementary data available online at the American Journal of Physiology–Lung Cellular and Molecular Physiology website.
targeting. Scrambled siRNAs were used as controls. Introduction of siRNA into the cells was by Effectene. Protein knockdown was monitored by immunoblotting.

Sialylation of GM1 in CF lung epithelial cells, P. aeruginosa adherence to CF lung epithelial cells, and NF-κB activation, αGM1 levels were assessed with fluorescently labeled cholera toxin, bacterial adhesion by colony counts, and NF-κB activation by luciferase assays, as previously described (38). CF and normal cells, grown postconfluence, were incubated for 48 h with 50 μM zmparin or 300 nM sildenafil. Cells were fixed and stained with fluorescently labeled cholera toxin B for 60 min at room temperature. Relative fluorescence was calculated from actual gray levels of 10 random fields from three independent experiments. Postconfluent cells, treated with 50 μM zmparin or 300 nM sildenafil, were incubated with P. aeruginosa (PAO1) at a multiplicity of infection of 1:200 in MEM media without serum or antibiotic supplements. Samples were washed three times with PBS and lysed with 0.5% TX-100. Cell associated bacteria were plated on P. aeruginosa isolation agar and colonies counted. Treatment with Pseudomonas pro-inflammatory lipopeptide (lipotocin) LptA and NF-κB activation analysis were carried out as previously described (15).

CFTR transgenic mice mucosal tissue short-circuit current measurements in ex vivo experiments with Ussing chamber mounts. Nasal mucosa was from adult F508del Cfp<sup>pm1/Em</sup> mice (congenic FVB, with residual CFTR currents) or Cfr<sup>−/−</sup> mice (congenic FVB, Cfr knockout with no CFTR currents) (43, 48). The chambers were filled with gassed (95% O<sub>2</sub>-5% CO<sub>2</sub>) Myllrer solution and the short-circuit current (Isc) measurements were started.

Animal model of P. aeruginosa aerosol infection and myeloperoxidase levels in lung tissues. DBA/2 mice (6–8 wk; Charles River Laboratories) were fed a specially formulated diet with or without sildenafil: 600 g of dry regular NIH mouse chow blended to coarse powder with sildenafil embedded in 1% agar paste frozen in ice-cube trays. P. aeruginosa aerosol infection model was carried out as previously described (33). Lung tissue-associated myeloperoxidase activity was measured in homogenized lung samples o-dianisidine dichloride oxidation assay (6). One unit of MPO activity is defined as the decomposition of 1 μmol of H<sub>2</sub>O<sub>2</sub>/min.

Statistics. Unless otherwise specified, statistical analyses were carried out using Fisher’s protected least significant difference post hoc test (ANOVA) (SuperANOVA, Abacus Concepts).

Additional methods. Additional methods are available online at the American Journal of Physiology–Lung Cellular and Molecular Physiology website.

RESULTS

Hyperacidification of TGN in CF respiratory epithelial cells is due to abnormally low inducible NO synthase and NO<sub></sub> levels. The levels of reduced NO in the CF lung are lower than normal (19, 25, 32). We tested the hypothesis that the NO<sub></sub> defect in CF respiratory epithelial cells contributes to TGN hyperacidification via a NO<sub></sub>-guanylate cyclase-cGMP cascade controlling sodium channel activity. The TGN pH was determined using the pH-sensitive GFP, as previously described (38), with Fig. 2A showing the characteristic perinuclear localization of the TGN38-pHlourin GFP probe to the TGN, in keeping with prior marker colocalization studies (38). The CF lung epithelial cell line IB3-1 (55), lacking in Cl<sup>−</sup> transport (confirmed using halide-sensitive YFP; supplemental Fig. S1) and expressing less inducible NO synthase (iNOS) than the genetically matched CFTR-corrected S9 cells (Fig. 2B–D), was treated with the NO<sup>−</sup>-donors DETA-NONOate and NOR-4. This normalized the pH in the TGN (DETA-NONOate: pH 7.0 ± 0.2; NOR-4: pH 6.9 ± 0.2) (Fig. 2E). Conversely, when S9 cells were incubated with NOS inhibitor L-NAME or highly specific iNOS inhibitor 1400W at 10 μM for 20 min (n = 5). G: CF cells (IB3-1) treated with guanylate cyclase agonists: 10 μM guanylin for 30 min, 10 μM protoporphyrin IX for 90 min, or 10 μM BAY41–2272 for 60 min (n = 5). Guanylate cyclase antagonists, 10 μM ODQ and NS-2028, for 30 min counteracted pH correction by 0.1 mM DETA-NONOate (n = 5). F: CFTR-corrected S9 cells were treated with 1 mM NO<sub></sub> inhibitor L-NAME for 60 min or its inactive stereoisomer R-NAME, or with specific inducible NOS (iNOS) inhibitor 1400W at 10 μM for 20 min (n = 5). E: CF cells (IB3-1) treated with guanylate cyclase agonists: 10 μM guanylin for 30 min, 10 μM protoporphyrin IX for 90 min, or 10 μM BAY41–2272 for 60 min (n = 5). Guanylate cyclase antagonists, 10 μM ODQ and NS-2028, for 30 min counteracted pH correction by 0.1 mM DETA-NONOate (n = 5). H: membrane permeant cGMP analogs 100 μM 8-Br-cGMP (60 min) and dibutyryl-cGMP (30 min) corrected TGN pH in IB3-1 cells (n = 5).

The signaling cascade responsible for TGN hyperacidification in CF includes guanylate cyclase. Further delineation of the pathway acting downstream of NO<sup>−</sup> indicated that guanylate cyclase was involved in TGN acidification. Incubating IB3-1 cells with guanylin, a small peptide physiologically present in normal lung fluids and acting as agonist of mem-
brane-bound guanylate cyclase, elevated the pH of the TGN in CF cells to 7.1 ± 0.4 (Fig. 2). Proporphyrin IX, an agonist of soluble guanylate cyclase, also increased TGN hyperacidification in IB3-1 cells (pH 7.4 ± 0.5). BAY41-2272, another soluble guanylate cyclase agonist, corrected the pH to 6.7 ± 0.1. Conversely, when the effects of the guanylate cyclase antagonists ODQ and NS-2028 were assessed in the presence of the NO-donor DETA-NONOate, both ODQ (pH 6.1 ± 0.1) and NS-2028 (pH 6.1 ± 0.2) counteracted normalization of TGN pH in response to DETA-NONOate (Fig. 2).

**Stimulation with cGMP normalizes TGN pH in CF cells.** We next tested whether cGMP can normalize the TGN pH in CF cells. Two membrane permissive forms of cGMP, 8-Br-cGMP and dibutyryl-cGMP, corrected the hyperacidification of TGN: the pH changed from 6.1 ± 0.1 to 6.9 ± 0.2 (for 8-Br-cGMP) and to 6.8 ± 0.2 (for dibutyryl-cGMP) (Fig. 2H). Collectively, these experiments connect the NO-deficiency in CF (19, 24, 32) to organellar hyperacidification in CF respiratory epithelial cells (9, 37, 38, 40) via a guanylate cyclase signaling pathway.

Cyclic GMP-specific phosphodiesterase inhibitors correct TGN hyperacidification in CF. Based on these observations, inhibiting cellular phosphodiesterases and increasing intracellular cGMP appeared as a viable approach for pharmacological intervention in CF. Of the 11 recognized PDE families, 7 are expressed in the lung, including PDE1, PDE3-7, and PDE9 (4, 17). The cGMP-specific PDE5 is abundant in the lung and, along with the cAMP-specific PDE4 (20), represents the predominant PDE form, accounting for nearly 80% of the overall PDE activity in the airways. When IB3-1 CF cells were treated with rolipram, an inhibitor specific for PDE4 (4, 17, 20) that hydrolyzes cAMP, no change in the TGN pH was observed (Fig. 3A). Zaprinast, a precursor in the development of the drug sildenafil, an inhibitor of PDE5 (which hydrolyzes cGMP), corrected TGN hyperacidification from pH 6.1 ± 0.2 to 6.9 ± 0.1 (Fig. 3A). At higher concentrations, zaprinast inhibits PDE6 and 9 (44). Increasing zaprinast concentration led to no additional change in pH relative to the PDE5-specific action at lower zaprinast concentrations (Fig. 3A), thus suggesting that PDE6 and PDE9 had no dominant effect. The role of PDE5 was further confirmed by using PDE5-inhibitor MBCQ, which corrected TGN hyperacidification in CF cells from 6.1 ± 0.2 to 6.8 ± 0.1 (Fig. 3A). IBMX, a nonselective PDE inhibitor, with added complexity of adenosine action, increased TGN pH from 6.1 ± 0.2 (untreated control) to 6.6 ± 0.3. The chloride channel activity of CFTR is readily activated by cAMP (46); thus, in this series of experiments, we could not rule out that the effect with IBMX could be attributed to cAMP rather than to cGMP.

To confirm our pharmacological analyses, we established by immunoblotting that PDE5 is expressed in human bronchial epithelial cells (Fig. S2). Next, PDE5 knockdown using siRNA was performed. As predicted, PDE5 siRNA, but not a scrambled control siRNA, corrected TGN pH in IB3-1 cells (Fig. 3A; pH 6.7 ± 0.1 and 6.0 ± 0.1, respectively; P = 0.0008). The results with PDE inhibitors were confirmed in additional cell lines. A tracheal epithelial cell line 9HTEc−, displaying CF phenotype due to the expression of a dominant negative CFTR construct (pCep-R) (7), was treated with zaprinast (Fig. 3B). The TGN pH was corrected (pH 6.2 ± 0.1 to 6.7 ± 0.2) and matched that in the normal control (9HTE− cells transfected with the plasmid pCep without the CFTR R-domain).

**Sildenafil efficiently corrects hyperacidification in primary human epithelial cells.** Sildenafil has shown promise in clinical studies for treatment of lung disease, such as primary pulmonary hypertension (42, 45). We tested this widely used FDA-approved PDE5 inhibitor, with a 240-fold greater specificity toward PDE5 than zaprinast. Sildenafil, at therapeutically relevant concentrations of 300 nM, normalized the TGN pH in IB3-1 cells, matching that of the CFTR-corrected S9 cells (Fig. 3, A and E–G). The correction of TGN hyperacidification on sildenafil treatment was confirmed in bronchial epithelial cell line CFBBe41o derived from a patient homozygous for-F508 CFTR mutation (32) (Fig. 3C). Moreover, primary human bronchial epithelial cells (CFTR F508/D/F508) obtained from lung transplants showed pH correction on sildenafil treatment (Fig. 3D).

**Sodium transport via ENaC is responsible for TGN hyperacidification in CF.** We have previously demonstrated (37, 38) that increased Na⁺ efflux from the TGN lumen in CF cells is responsible for the hyperacidification of this organelle in a
CFTR-defective background (see model in Fig. 1). In CF cells, the open probability of ENaC is increased, allowing efflux of Na\(^{+}\), which is delivered into the organellar lumen by Na\(^{+}\)-K\(^{+}\)-ATPase. Free Na\(^{+}\) efflux in CF organelles dissipates the membrane potential, thus delaying the normal physiological inhibition of the v-H\(^{+}\)ATPase proton pump based on positive charge build-up (37, 38). As a net result, the TGN lumen becomes hyperacidified in CF cells.

First, we confirmed that ENaC colocalizes with the TGN38-GFP probe (Fig. 4, A–D) and is expressed at equal levels in CF and normal respiratory cells, as shown by immunoblots (Fig. 4E). We established the sequential aspects of this model, which predicts that cGMP corrects TGN hyperacidification in CF cells via ENaC, since cGMP is a potent amiloride-sensitive sodium channel blocker (29). Sildenafil increased cGMP (Fig. 5A) but not cAMP (Fig. 5B) levels in primary CF lung epithelial cells. Inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase using the membrane-permeant inhibitor acetylstrophanthidin (37, 38) induced hyperacidification in CFTR-corrected cells (Fig. 5C). A membrane permeant inhibitor of ENaC, benazamil, corrected the TGN hyperacidification in IB3 cells (Fig. 5D) and in primary CF cells homozygous for the F508 CFTR allele (Fig. 5E).

Finally, we demonstrated that ENaC was responsible for these effects using ENaC-α siRNA. ENaC-α knockdown (Fig. 5F) corrected the pH of the TGN in IB3-1 cells (Fig. 5G), whereas control siRNA treatment did not. In addition, we established that chloride channel activity of CFTR did not affect TGN pH, since inhibition of CFTR Cl\(^{-}\) transport by a highly selective channel inhibitor, CFTRinh-172 (30), did not alter organellar pH in normal cells (Fig. 5H).

PDE5 inhibitor sildenafil corrects pathological cascade in CF epithelial cells. A candidate drug must be able to ameliorate a set of defined pathological abnormalities in CF. The disease-causing effects of TGN hyperacidification in CF are the characteristic undersialylation of plasma membrane glycoproteins and glycolipids and increased adhesion of CF pathogens, e.g., P. aeruginosa, to asialo-glycoconjugates (7, 11, 22, 27, 38), which, at later stages of advanced disease, lead to P. aeruginosa association with the mucus (50). Zaprinast and sildenafil restored sialylation in IB3-1 cells to the levels seen in the CFTR-corrected S9 cells (Fig. 6, A–C). PDE5 inhibition also corrected sialylation in another tested CF cell line (CFBE410−), restoring it to the levels seen in 16HBE14o− normal human bronchial cells (32) (Fig. 6C). The effects of sildenafil could not be attributed to mislocalization of the sialyltransferase since it remained in the TGN (Fig. 6, D and E). Thus the correction of the sialylation defect is due to normalization of the pH at the appropriate site of sialyltransferase enzymatic action.
PDE5 inhibitors also corrected \( P. \) aeruginosa adhesion to CF cells. Bacterial adherence to IB3-1 cells was reduced to the levels seen with the CFTR-corrected S9 cells (Fig. 6F). CFBE410− cells treated with sildenafil showed a 10-fold reduction of bacterial adhesion, bringing it to levels comparable with normal bronchial cells (16HBE140−) (Fig. 6G). Similar reduction in \( P. \) aeruginosa adhesion was observed in 9HT-Eo− pCep-R cells treated with sildenafil (Fig. 6G). Reduction of \( P. \) aeruginosa adhesion on sildenafil treatment was confirmed in primary human-F508/F508 bronchial epithelial cells (Fig. 6H). Thus pharmacological inhibition of PDE5 corrects both the sialylation and bacterial colonization defects associated with CF respiratory epithelial cells (3, 7, 22, 38).

Another critical aspect of lung pathogenesis in CF is an exaggerated proinflammatory response to \( P. \) aeruginosa pilin (12) and lipoproteins (15). We tested the effect of sildenafil on \( P. \) aeruginosa pilin (12) and lipoproteins (15). We tested the effect of PDE5 inhibition on \( P. \) aeruginosa susceptibility to glibenclamide, and 3) the \( I_c \) response to sildenafil was absent in null \( CFTR \) transgenic mice and \( CFTR \) transgenic mice (congenic, FVB). Thus sildenafil effects on mucosal tissues from \( CFTR \) congenic mice indicate further benefits of Sildenafil treatment, beyond the effects on organellar pH, by enhancing CFTR currents.

### Sildenafil reduces neutrophil lung infiltration in mice aerogenously infected with \( P. \) aeruginosa

We tested the effects of sildenafil on relevant inflammatory parameters (neutrophil infiltration, which is a principal cause of lung damage in CF) in an animal model of respiratory exposure to \( P. \) aeruginosa aerosols (52) using DBA/2 mice sensitive to \( P. \) aeruginosa (34). No significant protection with sildenafil was detected against mortality in this model of acute infection (n = 90, \( P = 0.294; \) Log Rank test). Myeloperoxidase measurements showed reduction in neutrophil infiltrates by 42 ± 11%

### Table 1. In vivo and ex vivo effects of sildenafil on lung mucosal function

<table>
<thead>
<tr>
<th>Experimental Group (DBA/2 mice)</th>
<th>Myeloperoxidase, U/mg lung tissue</th>
<th>Value</th>
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<tr>
<td>Control</td>
<td>16.3 ± 1.8</td>
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<tr>
<td>Sildenafil</td>
<td>11.4 ± 1.2</td>
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In vivo and ex vivo effects of sildenafil on lung mucosal function: improvement of electrolyte currents in tissue explants from \( CFTR \) transgenic mice and reduced lung inflammation in aerosol infection model in \( P. \) aeruginosa-susceptible mice. Top: improved chloride currents across mucosal isolated from CF F508del \( Cfrtm1Eur \) congenic mice. Basal mucosa from adult F508del \( Cfrtm1Eur \) mice (congenic FVB, with residual CFTR currents) or \( CFTR \)−/− mice (congenic FVB, \( Cfr \) knockout with no CFTR currents). After equilibration, compounds were added in a standardized order to the mucosal (M) or serosal (S) side of the tissue: 1) indomethacin (10 mM, M+S), to reduce basal Cl− secretion caused by endogenous production of prostaglandins; 2) amiloride (10 mM, M), to inhibit amiloride-sensitive electrogenic Na+ absorption (reflecting ENaC activity); 3) sildenafil (1 mM, M+S), to inhibit PDE5 and raise intracellular cGMP levels; 4) amiloride was removed by repeated washings to de-inhibit ENaC; 5) sildenafil was again added (1 mM, M+S), and constant current was achieved; 6) amiloride (10 mM, M) was added to measure the ENaC activity in the presence of sildenafil. Assays were performed in duplicate. *N = 5 (F508del \( Cfrtm1Eur \), FVB congenic mice). **Sensitive to glibenclamide and equaling 42% of forskolin/cAMP activation (determined in separate experiments). ***Short-circuit current \( I_c \) response was absent/ negligible in null \( CFTR \)−/− transgenic mice (congenic, FVB). Bottom: reduced polymorphonuclear (PMN) lung monocyte lung infiltration in the lungs of \( P. \) aeruginosa-susceptible DBA2 mice placed on a regimen of sildenafil in the diet on aerosol-delivered respiratory infection with \( P. \) aeruginosa. PMN infiltration was monitored by myeloperoxidase assay in lung homogenates.
(n = 8, P = 0.047, t-test) at 12 h postexposure in sildenafil-treated animals (Table 1). Effects on mortality in chronic infection (avoiding methods of artificially imposed chronic infection, e.g., by embedding bacteria in agar beads) could not be monitored, since mice either succumbed to naturally delivered acute infection or cleared P. aeruginosa rapidly from the lungs as previously shown (52).

DISCUSSION

The experiments reported here connect the hitherto disparate defects in CF: the lower than normal NO levels (19, 25, 32) and the Na+ transport-dependent hyperacidification of the organellar lumen in CF (37, 38, 40). This work demonstrates, using ENaC knockdowns, the cascade involving NO, cGMP, and ENaC in organellar hyperacidification and dysfunction in CF. This study, along with recent indirect data indicating (39) that a similar pathway operates in the endosomal compartment in CF cells, establishes the cascade of signaling and physiological events explaining several of the principal pathological features in CF. As depicted in the model in Fig. 1C, the lower than normal NO levels in CF respiratory epithelial cells (19, 25, 32) cause suboptimal cGMP levels. The uninhibited ENaC, which is normally blocked by cGMP, results in enhanced Na+ efflux from the organellar lumen in CF. This in turn dissipates the positive charge build-up and allows the proton pump to work excessively, resulting in organellar hyperacidification. This molecular pathological cascade in CF lung cells can be corrected by interventions at several steps along the pathway: by adding exogenous NO2, by stimulating guanylate cyclase using agonists, by increasing intracellular cGMP with membrane permeant cGMP analogs, or by incubating the cells with cGMP-specific phosphodiesterase inhibitors. These data also suggest that blocking ENaC by elevating intracellular cGMP may correct the molecular pathological cascade seen in CF lung epithelia cells.

Our laboratory’s recent report (39) suggests that inhibition of PDE5 corrects hyperacidification of recycling endosomes in CF cells in a similar fashion to the processes reported here. It is likely that the pertinent signaling pathways, delineated in the present study for the TGN, apply to other organelles in which ENaC and sodium fluxes play a role, including endosomes in CF respiratory epithelial cells (40). Furthermore, a study by Dormer and colleagues has indicated that sildenafil affects F508-CFTR localization in nasal epithelial cells (13). The concentration of sildenafil used by Dormer et al. (13) ranged from 150 μM for correction of CFTR trafficking to 1 mM for correction of chloride channel activity. This exceeds by far the more pharmacologically relevant concentrations used in our study (300 mM sildenafil) that match plasma levels achieved in standard sildenafil use in humans. Nevertheless, the previously published studies (13, 39) and the present work point to the potentially beneficial action of sildenafil in CF.

Our study demonstrates that pharmacological inhibition of PDE5 corrects a succession of interconnected defects in CF respiratory epithelial cells. Moreover, the pathological manifestations of the pH defect (37, 38, 40) are corrected by sildenafil treatment. This includes 1) undersialylation of plasma membrane glycoconjugates, 2) bacterial adhesion, 3) excessive proinflammatory response, 4) electrolyte transport across respiratory mucosa, and 5) lung inflammation in response to CF-specific bacterial pathogen. The observation that a sequence of abnormalities in CF can be connected as shown in the present study indicates that a common root to the clinically relevant problems can be identified. It is equally important, that this chain of pathological consequences can be corrected orameliorated by clinically proven PDE5 inhibitors. Since this can be achieved with low, therapeutic doses of sildenafil as shown in the present study, our findings represent a step toward clinical trials with sildenafil in CF.

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