SUPPLEMENTARY INFORMATION

Supplementary Methods:

Cells and tissue culture. IB3-1 is a human bronchial epithelial cell line derived from a CF patient with the mutant CFTR genotype ΔF508/W1282X (Egan et al., 1992). S9 is a CFTR-corrected IB3-1 derivative stably transfected with a full size functional CFTR (Egan et al., 1992). CFBE41o- is a CF cell line derived from a patient with the CFTR genotype ΔF508/ΔF508, and 16HBEo- is a normal human bronchial epithelial cell line (Gruenert et al., 2004; Meng et al., 1998). IB3-1 and S9 cells were maintained in complete LHC-8 medium (Biosource Int., MD). CFBE41o- and 16HBEo- cells were maintained in complete DMEM (GibcoBRL, Life Technologies, MD). Primary normal human bronchial epithelial cells (NHBE) were from Clonetics. Primary CF lung epithelial cells from CF patient lung transplants were from P. Karp, and were maintained in supplemented BEBM (Camberx, MD). CFTR genotyping was performed by PCR and oligonucleotide ligation assay. All cells were grown in a humidified chamber at 37°C under 5% CO_2.

Transfections. The use of cellubrevin-GFP pHluorin (Miesenbock et al., 1998) in human respiratory epithelial cells cells was previously described (Poschet et al., 2002b). Cells were seeded onto 25 mm coverslips in 6 well plates at $1x10^5$ cells/ml and transfected with Effectene reagent (Quiagen, CA) with 1μg/ml cellubrevin GFPpHluorin. After 72 hours at 37°C and 5% CO_2, coverslips were mounted in perfusion chambers and visualized by live microscopy.

Chemicals. Sildenafil (Ballard et al., 1998) was prepared as previously described (Francis et al., 2003). The following chemicals were from BIOMOL Research Laboratories: *N*-nitro-L-arginine methyl ester (L-NAME), *N*-nitro-R-arginine methyl ester (R-NAME), (±)-3-(E)-Ethyl-2'-((E)-hydroxyimino-5-nitro-3-hexenecarbamoyl) pyridine (NOR-4), (Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate), protoporphyrin IX, 4H-8-bromo-1,2,4-oxadiazolo(3,4-d)benz(b)-(1,4)oxazin-1-one (NS-2028), 3-Isobutyl-1-methylxanthine (IBMX), 4-[[3,4-(Methy-lenedioxy)benzyl]amino]-6-chloroquinazoline (MBCQ), 8-Br-cGMP, dibutyryl-cGMP, 1H-[1,2,4]-Oxadiazolo-[4,3-α]-quinoxalin-1-one (ODQ) and N-(3-(Aminomethyl)benzyl)acetamidine•2Hcl (1400W). The following chemicals were from Sigma: 4-[3-(Cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone (Rolipram) and (3,β-Acetoxy-5α, 14-dihydroxy-19-oxo-20[22]-cardenolide) (Acetylstrophanthidin) and N-(Benzylamidino)-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride (Benzamil).

Confocal fluorescence microscopy for localization studies. Cells were transfected with cellubrevin GFP pHluorin as described above. After 72 h, cells were treated with 300 nM Sildenafil for 1 h, fixed in 2% paraformaldehyde, stained with mouse monoclonal anti-transferrin receptor antibody (Zymed, CA), and Alexa 568 anti-mouse secondary antibody (Molecular Probes, OR). Cells were visualized with a Zeiss 510 META microscope and images were processed by Zeiss LSM software (Carl Zeiss International).

Immunofluorescence analysis of iNOS. IB3-1 and S9 cells were grown until 80% confuency. Cells were fixed with 1% paraformaldehyde followed by membrane permeabilization using 1% TritonX-100. The coverslips were washed three times with PBS prior to incubating with blocking solution (10% skim milk, 6% BSA fraction V, and 2% goat serum in PBS). Airway epithelial cells were incubated with a polyclonal rabbit anti-iNOS antibody (Transduction Labs., Lexington, KY) at 4°C overnight at a dilution of 1:500, followed by a secondary antibody. Alexa 488-conjugated to goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) was diluted 1:1000 and incubated with samples for 5 h at 37°C in blocking solution. Coverslips were mounted on glass slides with PermaFluor (Immunon, Pittsburgh, PA) and analyzed using an Olympus IX70 microscope (Olympus, Melville, NY). All images were taken at a magnification of x100. Confocal fluorescence microscopy images were collected and quantified using the PerkinElmer Life Sciences UltraView confocal microscopy system.

Measurement of cGMP. Levels of cGMP were determined using Cyclic GMP EIA Kit (Biomedical Technologies Inc., Stoughton, MA). S9 and IB3-1 cells were grown to confluency in T25 flasks (approx. $3X10^6$ cells/flask). IB3-1 cells were treated with 300 nm sildenafil for 1 h. Cells were lysed in 5% trichloroacetic acid and extracted in 50% aqueous ether. Lysates were lyophilized dry and resuspended in 250 μL H_2O. An aliquot (4 μL) was used to determine protein concentration (BCA Protein Assay Kit, Pierce, Rockford, IL) and an aliquot (210 μL) was utilized to determine cGMP concentration following the acetylation protocol.

PDE5 siRNA knockdown and immunoblot analysis. IB3-1 cells were grown on 6-well glass coverslips to 80% confluency and transfected utilizing an Effectene kit (Quiagen, Valencia, CA) with siRNA specific for human PDE5 (SMARTpool, Dharmacon, Lafayette, CO). Transfections proceded for 48 h, and cells were lysed in CytoBuster solution (Novagen, San Diego, CA). Lysates were subjected to SDS-PAGE in a 10% gel (Bio Rad, Hercules, CA) and transferred for 1hr at 100 V to Criteron PVDF membrane (Bio Rad, Hercules, CA). Blots were hybridized with rabbit polyclonal anti-PDE5 antibody (Cell Signaling Technology, Inc., Danvers, MA) at 1:500 and mouse monoclonal anti-GAPDH antibody (Abcam, Cambridge, MA) at 1:1000 overnight at 4°C. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Pierce, Rockford, IL) were applied at 1:5000 for 2 hrs at room temperature, and signal was detected utilizing Supersignal reagent (Pierce, Rockford, IL). Blots were exposed to Hyperfilm chemiluminescent film (Amersham Biosciences, Buckinghamshire, UK) for 10 min. Autoradiograms were scanned and band intensity quantified using NIH Image J program.

ELISA. To analyze IL-8 protein expression, $2x10^4$ IB3-1 cells/well were seeded in a 96-well plate and cultured in complete LHC-8 medium overnight. Prear to treatment, cells were incubated in serum-free media for 21 h. After that, cells were incubated in the presence and absence of 25 μg/ml *P. aeruginosa* DNA for 24 h as a physiologically relevant source of unmethylated CpG motifs that elicit IL-8 response. IL-8 was measured in supernatants using an ELISA kit (R&D Systems, MN). When indicated, 300 nM Sildenafil was added to the cells 2 h before stimulation.

Statistics. All statistical analyses were carried out using Fisher's Protected LSD post hoc test (ANOVA) (SuperANOVA v1.11, Abacus Concepts, Inc., CA)

Supplementary Notes and References

Note S1. Organellar pH in CF was initially proposed to be alkaline based on a loss in CF cells of Cl^- transport via CFTR, as it was believed that efflux of negative charge could dissipate membrane potential thus assisting the proton pump; in that model, organelles in normal cells would be acidified, while, due to the loss of CFTR, CF cells would display more alkaline lumen (Barasch et al., 1991). Organellar alkalinization model was initially contested by Verkman and others who used a heterologous system in Swiss 3T3 fibroblasts and MDCK cells and reported that TGN was not alkalinized upon expression of the mutant form of CFTR relative to expression of a functional CFTR (Seksek et al., 1996). It is important to note that in such studies it was not possible to detect whether TGN was abnormally acidified in CF respiratory epithelial cells, since CFTR-ENaC relationships are highly cell type specific, and can range from inhibition to activation: CFTR in sweat glands activates sodium channels (Reddy et al., 1999) and negatively affects ENaC activity only in respiratory epithelial cells (Stutts et al., 1995). Subsequently, it was demonstrated directly in human respiratory epithelial cells that organellar pH was lower in CF bronchial and tracheal epithelial cells than in genetically matched CFTR-corrected cells (Chandy et al., 2001; Poschet et al., 2002a; Poschet et al., 2001; Poschet et al., 2002b). These relationships have been previously reviewed (Machen et al., 2001; Poschet et al., 2002a). See Fig. 5 in the main text for the current model.

Ballard, S.A., Gingell, C.J., Tang, K., Turner, L.A., Price, M.E. and Naylor, A.M. (1998) Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol*, 159, 2164-2171.

Barasch, J., Kiss, B., Prince, A., Saiman, L., Gruenert, D. and al-Awqati, Q. (1991) Defective acidification of intracellular organelles in cystic fibrosis. *Nature*, 352, 70-73.

Chandy, G., Grabe, M., Moore, H.P. and Machen, T.E. (2001) Proton leak and CFTR in regulation of Golgi pH in respiratory epithelial cells. *Am J Physiol Cell Physiol*, 281, C908-921.

Egan, M., Flotte, T., Afione, S., Solow, R., Zeitlin, P.L., Carter, B.J. and Guggino, W.B. (1992) Defective regulation of outwardly rectifying Cl- channels by protein kinase A corrected by insertion of CFTR. *Nature*, 358, 581-584.

Francis, S.H., Sekhar, K.R., Rouse, A.B., Grimes, K.A. and Corbin, J.D. (2003) Single step isolation of sildenafil from commercially available Viagra tablets. *Int J Impot Res*, 15, 369-372.

Gruenert, D.C., Willems, M., Cassiman, J.J. and Frizzell, R.A. (2004) Established cell lines used in cystic fibrosis research. *J Cyst Fibros*, 3 Suppl 2, 191-196.

Machen, T.E., Chandy, G., Wu, M., Grabe, M. and Moore, H.P. (2001) Cystic fibrosis transmembrane conductance regulator and H+ permeability in regulation of Golgi pH. *Jop*, 2, 229-236.
Meng, Q.H., Springall, D.R., Bishop, A.E., Morgan, K., Evans, T.J., Habib, S., Gruenert, D.C., Gyi, K.M., Hodson, M.E., Yacoub, M.H. and Polak, J.M. (1998) Lack of inducible nitric oxide synthase in bronchial epithelium: a possible mechanism of susceptibility to infection in cystic fibrosis. *J Pathol*, 184, 323-331.
Miesenbock, G., De Angelis, D.A. and Rothman, J.E. (1998) Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*, 394, 192-195.
Poschet, J., Perkett, E. and Deretic, V. (2002a) Hyperacidification in cystic fibrosis: links with lung disease and new prospects for treatment. *Trends Mol Med*, 8, 512-519.
Poschet, J.F., Boucher, J.C., Firoved, A.M. and Deretic, V. (2001) Conversion to mucoidy in Pseudomonas aeruginosa infecting cystic fibrosis patients. *Methods Enzymol*, 336, 65-76.
Poschet, J.F., Skidmore, J., Boucher, J.C., Firoved, A.M., Van Dyke, R.W. and Deretic, V. (2002b) Hyperacidification of cellubrevin endocytic compartments and defective endosomal recycling in cystic fibrosis respiratory epithelial cells. *J Biol Chem*, 277, 13959-13965.
Reddy, M.M., Light, M.J. and Quinton, P.M. (1999) Activation of the epithelial Na+ channel (ENaC) requires CFTR Cl- channel function. *Nature*, 402, 301-304.
Seksek, O., Biwersi, J. and Verkman, A.S. (1996) Evidence against defective trans-Golgi acidification in cystic fibrosis. *J Biol Chem*, 271, 15542-15548.
Stutts, M.J., Canessa, C.M., Olsen, J.C., Hamrick, M., Cohn, J.A., Rossier, B.C. and Boucher, R.C. (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science*, 269, 847-850.

Supplementary figures

Fig. S1. Decreased levels of iNOS in CF respiratory epithelial cells. A. Images show iNOS immunofluorescence in IB3-1 (CF) and S9 (CFTR-corrected) cells. B. Quantification (%) of iNOS immunofluorescence in CFTR-corrected cells. Data, means ± SEM (**$p < 0.01$).

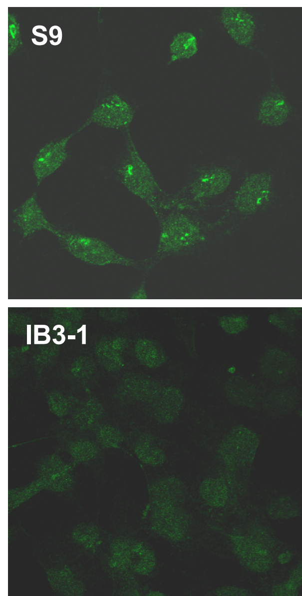
Fig. S2. PDE5 knockdown by siRNA in IB3-1 cells. **A**. Western blot using protein extracts from cells treated with PDE5 siRNA (lane 2) and controls (no siRNA, lane 1, or scrambled siRNA, lane 3). Blots were developed using antibodies to PDE5 (100 kD) and GAPDH (36 kD), with the latter serving as a loading control. **B.** Quantification of protein expression. $R_{PDE5/GAPDH}$: band intensity ratio between PDE5 and GPDH (used as a loading control): control, 0.248; PDE5 siRNA, 0.075; Scrambled siRNA, 0.240.

Fig. S3. Sodium transport dependence of endosomal hyperacidification in CF respiratory epithelial cells. A. Pharmacologically-induced hyperacidification in cellubrevin endosomes in CFTR-corrected cells and its correction with Sildenafil: S9,

pH 6.8±0.1, n=18; S9+10 μM Acetylstrophanthidin (inhibitor of Na^+/K^+ ATPase) pH 6.3±0.1, n=10, p=0.0015; S9+10 μM Acetylstrophanthidin+300 nM Sildenafil, pH 7.0±0.1, n=10, p=0.0523. **B.** Amiloride–sensitive sodium channels are involved in endosomal hyperacidification in CF cells. CF cells were treated with a membrane permeant ENaC-inhibitor, benzamil; IB3-1 control, pH 6.0±0.1, n=5, IB3-1+10 mM benzamil pH6.5±0.3, n=5, p=0.0402.

Fig. S4. Dose-dependent *P. aeruginosa* DNA stimulation of IL-8 secretion in human respiratory epithelial cells. IB3-1 cells were incubated with *P. aeruginosa* PAO1 DNA at indicated concentrations. IL-8 protein was measured in cell supernatants by ELISA.

Fig. S5. IL-8 levels in non-stimulated cells are unaffected by Sildenafil. IB3-1 cells were incubated for 24 h with media alone (open bar), 300 nM or 1000 nM Sildenafil (stripped bars) or 25 μg/ml of *P. aeruginosa* PAO1 DNA (gray bar) included as a positive control for IL-8 secretion in response to agonist stimulation. When Sildenafil was present, cells were pre-incubated with it for 2 h prior to stimulation. IL-8 was measure in supernatants by using an ELISA kit (R & D). Values represent means and standard errors. ** p<0.01 (ANOVA).



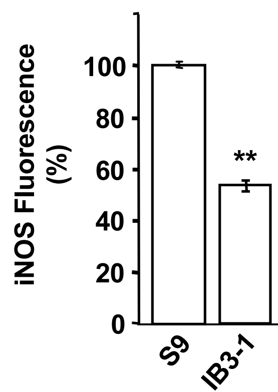
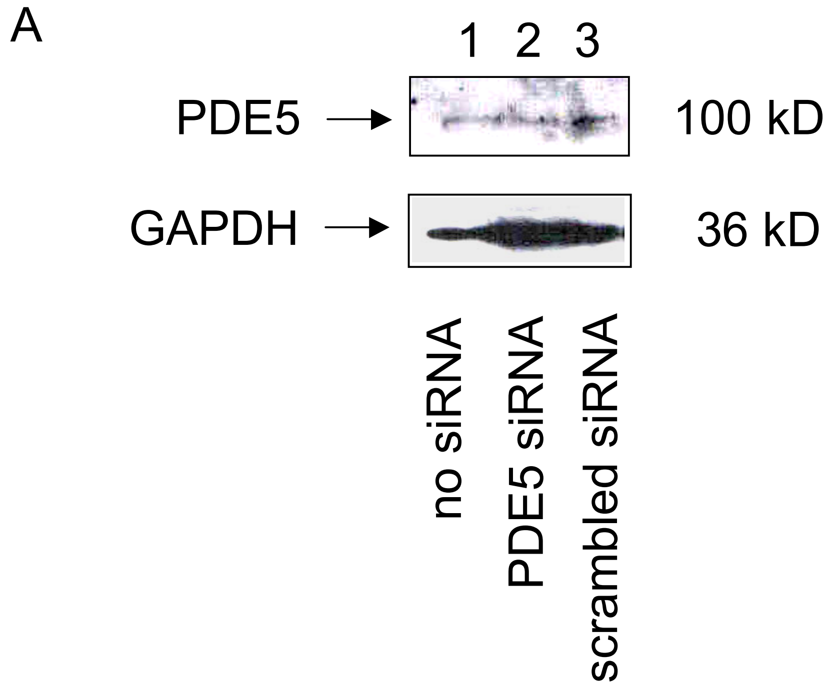
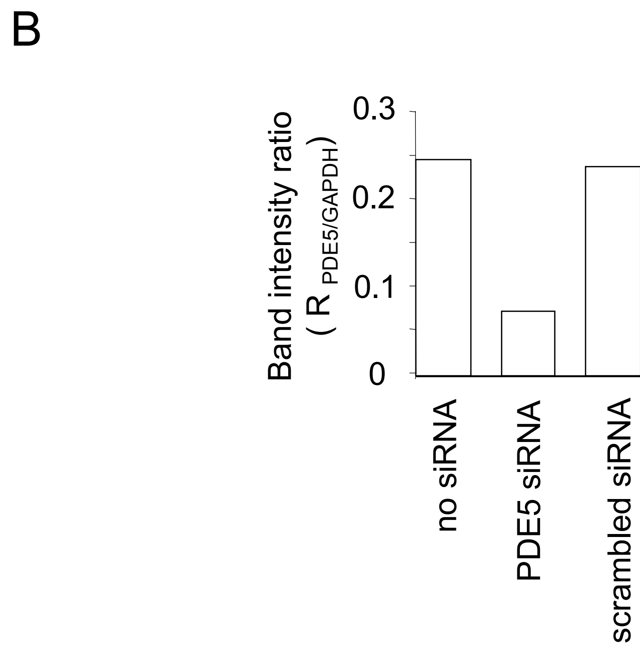


Fig. S1





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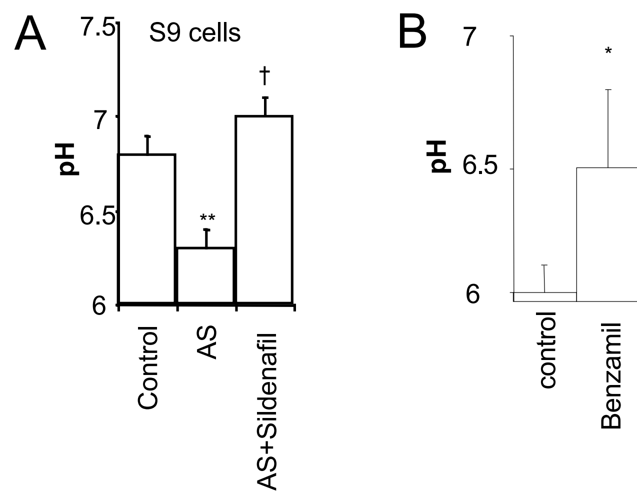

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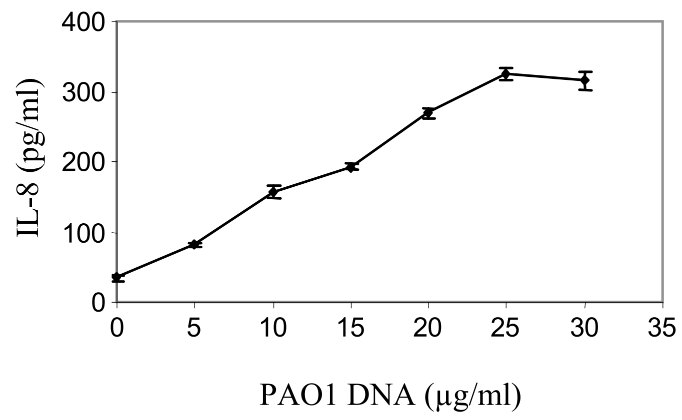

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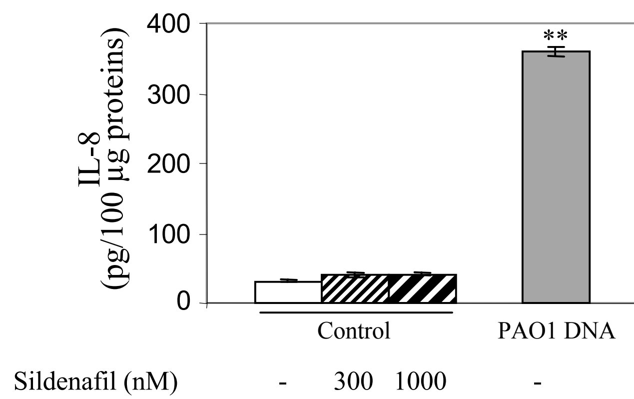

Fig. S5