

# In Vitro Pharmacologic Restoration of CFTR-mediated Chloride Transport with Sodium 4-Phenylbutyrate in Cystic Fibrosis Epithelial Cells Containing $\Delta$ F508-CFTR

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## Abstract

The most common cystic fibrosis transmembrane conductance regulator mutation,  $\Delta$ F508-CFTR, is a partially functional chloride channel that is retained in the endoplasmic reticulum and degraded. We hypothesize that a known transcriptional regulator, sodium 4-phenylbutyrate (4PBA), will enable a greater fraction of  $\Delta$ F508-CFTR to escape degradation and appear at the cell surface. Primary cultures of nasal polyp epithelia from CF patients ( $\Delta$ F508 homozygous or heterozygous), or the CF bronchial epithelial cell line IB3-1 ( $\Delta$ F508/W1282X) were exposed to 4PBA for up to 7 d in culture. 4PBA treatment at concentrations of 0.1 and 2 mM resulted in the restoration of forskolin-activated chloride secretion. Protein kinase A-activated, linear, 10 pS chloride channels appeared at the plasma membrane of IB3-1 cells at the tested concentration of 2.5 mM. Treatment of IB3-1 cells with 0.1–1 mM 4PBA and primary nasal epithelia with 5 mM 4PBA also resulted in the appearance of higher molecular mass forms of CFTR consistent with addition and modification of oligosaccharides in the Golgi apparatus, as detected by immunoblotting of whole cell lysates with anti-CFTR antisera. Immunocytochemistry in CF epithelial cells treated with 4PBA was consistent with increasing amounts of  $\Delta$ F508-CFTR. These data indicate that 4PBA is a promising pharmacologic agent for inducing correction of the CF phenotype in CF patients carrying the  $\Delta$ F508 mutation. (*J. Clin. Invest.* 1997. 100:2457–2465.) Key words: cystic fibrosis • CFTR • phenylbutyrate • pharmacotherapy

## Introduction

Cystic fibrosis (CF)<sup>1</sup> is a systemic disorder that results when mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an apical membrane glycoprotein, lead to a reduction in apical membrane chloride transport. CFTR is a cAMP-dependent chloride channel that regulates fluid compo-

sition in the respiratory and gastrointestinal tracts. In CF, unusually thick mucus leads to recurrent airway and intestinal obstruction, as well as chronic respiratory infection.

70% of the mutant CFTR alleles in the Caucasian population result from deletion of phenylalanine at position 508 ( $\Delta$ F508-CFTR). This mutation results in a protein capable of conducting chloride (1–4), but absent from the plasma membrane because of aberrant intracellular processing (5). Under usual conditions at 37°C,  $\Delta$ F508-CFTR is retained in the endoplasmic reticulum (ER), perhaps by prolonged association with the ER chaperones calnexin (6) and hsp70 (7), and is then targeted for degradation by the ubiquitin/proteasome pathway (8, 9). There is one published report that by overexpression of  $\Delta$ F508-CFTR, functional  $\Delta$ F508-CFTR protein can be measured at the cell surface. This work used  $\Delta$ F508-homozygous CF airway and immortalized pancreatic cells treated with the transcriptional regulator butyrate (10), a prototype drug with considerable toxicity and a half-life necessitating intravenous administration (11).

The  $\Delta$ F508 “trafficking” block is also reversible by incubation of cultured CF epithelial cells at reduced temperatures (25–27°C). In a time-dependent manner, lowered temperature results in the appearance of CFTR protein and channel activity at the cell surface (12). This observation suggests an intrinsic thermodynamic instability in  $\Delta$ F508-CFTR at 37°C that leads to recognition of the mutant protein by the ER quality control mechanism, prevents further trafficking, and results in protein degradation. Thermal stabilization may allow the protein to assume the appropriate conformation for normal trafficking. High concentrations of glycerol (1 M or 10%), a protein stabilizing agent or “chemical chaperone,” also appears to facilitate movement of  $\Delta$ F508-CFTR from the ER to the plasma membrane (13, 14).

Our goal is to identify nontoxic, clinically useful compounds that will increase the amount of  $\Delta$ F508-CFTR on the surface epithelium of patients with CF. Sodium 4-phenylbutyrate (4PBA) is a butyrate analogue that is approved for clinical use as an ammonia scavenger in subjects with urea cycle disorders. It is also a known transcriptional regulator of  $\beta$ - and  $\gamma$ -globin (15) that may act by inhibiting histone deacetylase (16). The hypothesis of this study is that 4PBA will promote  $\Delta$ F508-CFTR trafficking to the cell surface.

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1. *Abbreviations used in this paper:* CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator;  $\Delta$ F508-CFTR, cystic fibrosis transmembrane conductance regulator containing a deletion of phenylalanine at position 508; ER, endoplasmic reticulum; 4PBA, sodium 4-phenylbutyrate; PKA, cAMP-dependent protein kinase; W1282X, mutation of tryptophan at position 1282 to a termination codon.

## Methods

**Cell culture.** Primary CF nasal epithelial cells were isolated from surgical specimens and grown in tissue culture flasks coated with vitronectin/fibronectin/bovine serum albumin using modifications of previously described procedures (17). IB3-1 cells (17) were grown on uncoated plastic supports or, for immunocytochemistry, on coated glass cover slips. Cells were routinely cultured in 5% CO<sub>2</sub> incubators in LHC-8 (Biofluids, Inc., Rockville, MD) supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin/streptomycin (GIBCO BRL, Gaithersburg, MD), 0.2 mg/ml Primaxin (imipenim; Merck and Co., West Point, PA), 80 µg/ml tobramycin (Eli Lilly & Co., Indianapolis, IN), and 2.5 µg/ml fungizone (Biofluids, Inc.). Cells for control experiments were cultured under routine conditions described above. Growth medium for the treated cells was comprised of the indicated agent at the indicated concentration added to the routine growth medium and incubated in a 5% CO<sub>2</sub> atmosphere.

**Chloride efflux.** Chloride efflux experiments were performed essentially as described by Trapnell et al. (18). Briefly, cells were seeded in coated 35-mm dishes under the indicated conditions and grown to 80–90% confluence. Cells were loaded with 3 µCi/dish of Na<sup>36</sup>Cl under equilibrium conditions in bicarbonate-free Ringer's solution buffered with 10 mM Hepes, pH 7.4, for 2–4 h at 37°C for control and 4PBA-treated cells and at 27°C for cells incubated at the 27°C condition. Cells were washed three times with 1 ml of ice cold Ringer's solution before addition of 1 ml Ringer's solution with (stimulated) or without (basal) 13.5 µM forskolin (Sigma Chemical Co.) at room temperature. The Ringer's solution was changed every 15 s and counted by liquid scintillation in CytoScint (ICN Research Products, Costa Mesa, CA). Cells were lysed after 3 min with 0.2 N NaOH and residual <sup>36</sup>Cl was determined by liquid scintillation. Apparent first-order rate constants for chloride secretion were determined from three to four independent time courses for each condition using a nonlinear least squares method. Statistical comparison of these rate constants was performed using a two-tailed *t* test.

**Single channel/patch clamp recording.** Single channel patch clamp studies were performed in the excised inside-out patch configuration using conventional procedures (19). Recording pipettes were constructed from borosilicate glass capillaries (Garner Glass, Claremont, CA) using a microelectrode puller (PP83; Narishige Scientific Instrument Laboratory, Tokyo, Japan). The pipettes were partially filled with a standard pipette solution and had tip resistance of 2–5 MΩ. Recording was performed at room temperature (20–22°C). Single channel currents were recorded with a patch clamp amplifier (Axopatch 200A; Axon Instruments Inc., Foster City, CA), low pass filtered at 1 kHz using an 8-pole Bessel filter, and stored on videotape after pulse code modulation (model PCM-501ES; Sony, Tokyo, Japan). Data were redigitized (4 kHz) for analysis, transferred to a desktop computer, and analyzed using pCLAMP6 (Axon Instruments Inc.).

The bath solution contained (mM): NaCl, 140; MgCl<sub>2</sub>, 2; EGTA, 1; Hepes, 5; and CaCl<sub>2</sub>, 0.5 (free Ca<sup>2+</sup> 110 nM as measured by Fura-2), pH 7.3. The pipette solution contained (mM): NaCl, 140; CaCl<sub>2</sub>, 2; and Hepes, 5, pH 7.3. All solutions were filtered through 0.2-µm filters.

Chloride channels were activated by the addition of the catalytic subunit of protein kinase A (PKA; Promega, Madison, WI) and Mg-ATP at final concentrations of 50 nM and 1 mM, respectively. To prevent CFTR channel rundown in excised patches, 1 mM Mg-ATP was added to the bath solution.

**Antibodies.** Antisera 169 and 181 directed against human CFTR peptides in the R domain and before NBF-1, respectively, were generated in rabbits and described previously in publications from our laboratory (20). Antiserum 169 was used at a 1:500 dilution for immunocytochemistry and antiserum 181 was used at 1:2,000 dilution for immunoblot detection. The secondary antibody for both immunocytochemistry and immunoblot analyses was a donkey anti-rabbit IgG-

horseradish peroxidase conjugate (Amersham, Arlington Heights, IL) used at 1:1,000 dilution for immunocytochemistry and 1:10,000 dilution for immunoblots. Incubation times for both primary antisera and secondary antibodies were 1 h at room temperature.

**Immunoblot analysis.** Whole cell lysates were prepared by solubilization with 2 or 10% SDS at 95°C as noted in the figure legends. Protein concentration in the lysates was determined using the Bio-Rad D<sub>C</sub> assay reagents with bovine plasma γ-globulin as a standard (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were resolved by electrophoresis on 5 or 6% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and immunodetection performed as previously described (20). Nonspecific binding was blocked by incubation of the nitrocellulose with 2% gelatin. Primary antisera and secondary antibodies were applied in buffer containing 0.4% bovine serum albumin for 1 h at room temperature. Detection of immunoreactivity was performed with the ECL chemiluminescence reagent (Amersham) and fluorography. Densitometry of these fluorographs was performed using an image digitizer and software (Ultra-Lum, Inc., Carson, CA) and analyzed using AAB 2-D Main software (Advanced American Biotechnology, Fullerton, CA).

**Immunocytochemistry.** Cells were grown on coated cover slips in 6-well plates under the indicated conditions in standard media. Cells were fixed in 10% formalin in phosphate-buffered saline and permeabilized with 95% methanol in phosphate-buffered saline. Immunocytochemical detection of CFTR with antiserum 169 was performed as above and as previously described (20). Histomark orange and true blue peroxidase (both from Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used according to the manufacturer's protocol to detect positive immunocytochemical reaction. Counterstain, when used, was Nuclear Fast red (Digene, Beltsville, MD) and was applied for 1–1.5 min. Samples on cover slips were dehydrated through sequential ethanol before mounting on slides with Mount Quick (Research Products International, Mount Prospect, IL). Photomicrographs were made using a Zeiss microscope (Zeiss, Oberkochen, Germany) and Kodak (Rochester, NY) T-64 slide film. Immunocytochemistry on control and 4PBA-treated cells was performed using the same antisera dilutions, incubation times, counter-staining times, exposure conditions, and printing conditions.

**Reagents.** Pharmaceutical grade 4PBA, manufactured by Triple Crown America (Perkasie, PA), was a gift from Dr. Saul W. Brusilow (Department of Pediatrics, The Johns Hopkins Hospital). Reagent grade butyric acid was purchased from Sigma Chemical Co. Glycerol, ACS reagent grade, was purchased from J.T. Baker (Phillipsburg, NJ) and was sterilized by autoclaving before use. Nitrocellulose was purchased from Schleicher and Schuell (Keene, NH). Electrophoresis grade chemicals were purchased from Bio-Rad Laboratories. All other reagents were of reagent grade or better.

## Results

**4PBA promotes functional correction of cAMP-mediated chloride transport in CF airway epithelial cells.** In normal epithelial cells, elevations in cAMP stimulate chloride transport by CFTR via phosphorylation by PKA and ATP binding. This is not observed in epithelial cells from CF patients in response to cAMP or cAMP-stimulating agents. ΔF508-CFTR forms PKA-activated chloride channels in lipid bilayers (1), in oocytes (2, 3), and in the ER membrane (4), but is prevented from reaching the epithelial cell surface (5). To study ΔF508-CFTR trafficking, we chose the IB3-1 cell line, an immortalized CF bronchial epithelial cell line with genotype ΔF508/W1282X (17). As is typical of CF epithelial cells, IB3-1 cells do not increase chloride transport in response to cAMP stimulation (18). The W1282X mutation produces an unstable, and therefore untranslated mRNA (21–24). All CFTR protein in

**Table I. Comparison of Apparent First Order Rate Constants for Chloride Efflux**

Condition	$k_{app}$ (min <sup>-1</sup> ) for Cl <sup>-</sup> efflux (mean±SEM)		<i>P</i> value (two-tailed <i>t</i> test)
	Basal	Forskolin (cAMP)	
37°C (control)	0.258±0.015	0.291±0.024	0.39
100 μM 4PBA, 37°C	0.304±0.044	0.474±0.018	0.017*
27°C	0.187±0.019	0.325±0.016	0.011*

Apparent first order rate constants of chloride efflux were determined for three to four independent time courses by a nonlinear least squares method. Rate constants were averaged and are expressed as mean±SEM for unstimulated (basal) and forskolin-stimulated chloride efflux for each condition of incubation. Statistical significance was determined using a two-tailed *t* test. \**P* < 0.05.

IB3-1 cells is ΔF508-CFTR which is retained in the ER and is undetectable at the cell surface.

Table I contains the apparent first order rate constants for radioisotopic chloride secretion from untreated and 4PBA-treated IB3-1 cells. As expected, IB3-1 cells grown in control medium at 37°C did not demonstrate an increased rate of chloride secretion in response to forskolin treatment. However, IB3-1 cells grown at 27°C (as a positive control) or in the presence of 100 μM 4PBA at 37°C for 3 d demonstrated a 1.6-fold increase in the apparent first order rate of chloride secretion in response to forskolin. In a separate experiment, treatment of IB3-1 cells with 2 mM 4PBA for 3 d led to a 1.35-fold increase in the rate of forskolin-stimulated chloride secretion (0.283±0.023 min<sup>-1</sup> stimulated vs. 0.210±0.021 min<sup>-1</sup> basal, mean±SEM, *n* = 3 in each group, *P* = 0.079 by two-tailed *t* test). These data are consistent with 4PBA facilitating the appearance of cAMP-stimulated chloride channels at the cell surface of CF epithelial cells.

We next confirmed that the observed cAMP-stimulated increases in IB3-1 cell chloride secretion after 4PBA treatment were due to CFTR chloride channels at the cell surface, and not alternative chloride channels. We examined single channel recordings in patches excised from IB3-1 cells grown at 37°C in the presence or absence of 4PBA (Table II). To maximize our ability to detect CFTR channels at the cell surface after 4PBA treatment, IB3-1 cells were grown in the presence of a single, high concentration (2.5 mM) of 4PBA for 2 d. These cells demonstrated chloride currents consistent with that of CFTR, namely a PKA-stimulated linear current/voltage profile with 10 pS conductance that was present in 9/15 patches. A representative patch recording and a current/voltage (*I/V*) plot summarizing these channel data are shown in Fig. 1. The open

**Table II. Single Channel Patch Clamp Analysis**

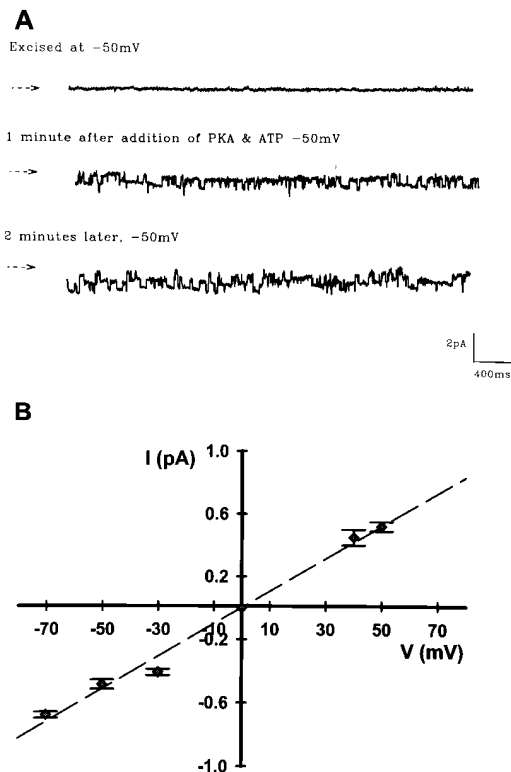
Cell type	Incubation conditions	Patches with CFTR channel activity
IB3-1	37°C	0/10* (0/15) <sup>‡</sup>
IB3-1	37°C, 2.5 mM 4PBA	9/15*
IB3-1	27°C	54/67 <sup>‡</sup>

\**P* = 0.0115 by two-tailed Fisher exact test. <sup>‡</sup>Data from Egan et al. (19).

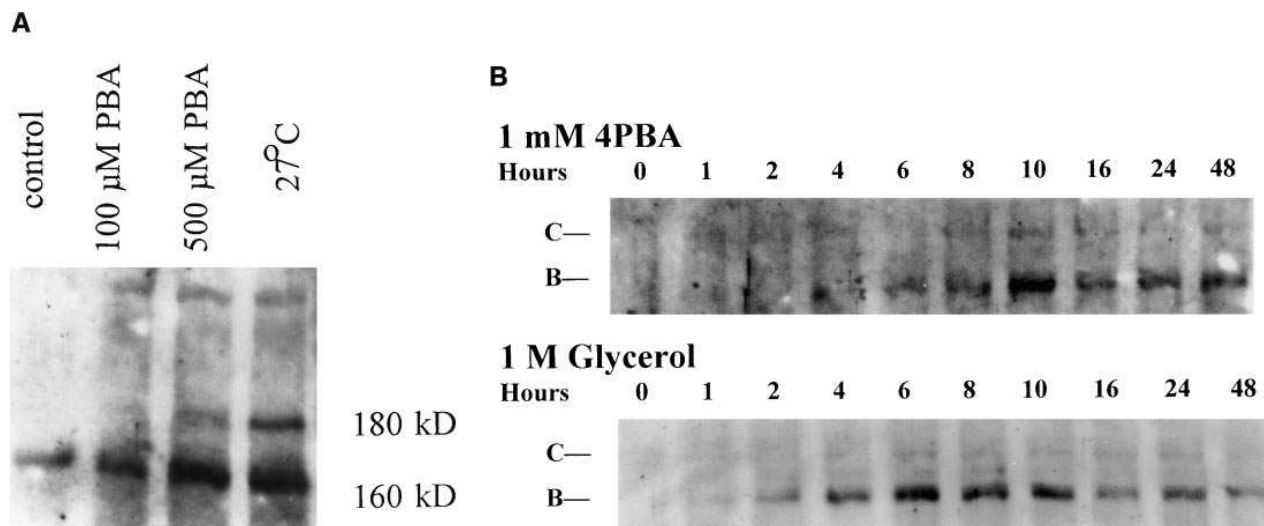
probability (*P*<sub>o</sub>) of these channels was 0.201±0.04, which is similar to that obtained in IB3-1 cells grown at reduced temperature (0.21±0.04) (19). This *P*<sub>o</sub> is intermediate to that previously reported for wild-type CFTR (0.35) and ΔF508-CFTR (0.1) on the cell surface (25), and similar to that of ΔF508-CFTR found on the ER membrane (0.16) (4). These comparisons suggest that ΔF508-CFTR channels in IB3-1 cells retain altered kinetics of chloride conduction, which would not be predicted for the W1282X allele.

Because these experiments were conducted in an excised patch configuration, we were unable to directly measure channel number. However, the frequency with which we detected CFTR activity in excised patches (9/15) is similar to that of IB3-1 cells incubated at reduced temperatures (57/81) (19), transformed fetal human tracheal epithelial cells in culture (56FHTE80<sup>-</sup>, no known CFTR mutation, 8/12) (19), and in transfected IB3-1 cells stably expressing wild-type CFTR (S9 cells, 8/10) (19). These observations suggest similar channel density in IB3-1 cells after 4PBA treatment as in corrected IB3-1 cells and non-CF tracheal epithelial cells.

The chloride conductance of these channels was blocked by 0.1 mM glibenclamide (*n* = 3), but was unaffected by 0.5 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, *n* = 2). This response to chloride channel blockers is typical of CFTR chloride channel activity. CFTR channel activity was not observed in plasma membrane patches from IB3-1 cells



**Figure 1.** Single channel recordings from IB3-1 cells grown in 4PBA. IB3-1 cells were cultured in the presence of 2.5 mM 4PBA for 2 d before single channel recording performed as described in Methods. (A) A representative patch recording of a CFTR channel. (B) A current/voltage plot of CFTR channel activity. Each point is the average of at least five determinations. The conductance of these channels was 10.3±0.05 pS and the open probability was 0.201±0.04.



**Figure 2.** Immunoblot of cell lysates from IB3-1 cells grown in the presence of 4PBA demonstrate shifts in the apparent mobility of CFTR consistent with trafficking through the Golgi apparatus. IB3-1 cells were (A) grown for 3 d under the indicated condition or (B) grown with 1 mM 4PBA or 1 M glycerol for the indicated time. Whole cell lysates were prepared by solubilization in 2% SDS at 95°C. 10  $\mu$ g of total protein was resolved on a 5% SDS-polyacrylamide gel before electrophoretic transfer to nitrocellulose for immunodetection of CFTR as previously described. The primary antiserum was a rabbit anti-prenucleotide binding domain 1 peptide antiserum (antiserum 181) and the secondary antibody was a donkey anti-rabbit horseradish peroxidase conjugate. Immunoreactivity was detected using the ECL reagent as described in Methods.

grown under control conditions at 37°C in the absence of 4PBA ( $n = 10$ ). Overall, these channel data in 4PBA-treated IB3-1 cells are similar to those obtained when IB3-1 cells were grown at reduced temperatures (19).

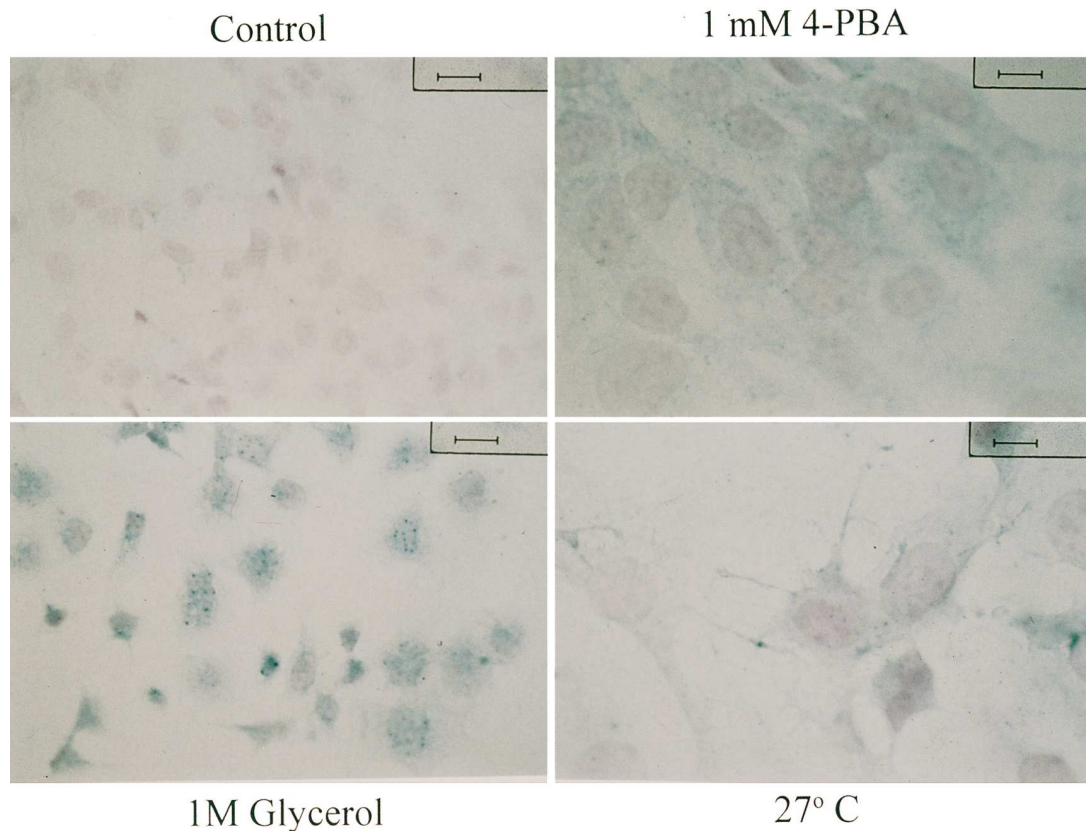
**4PBA treatment leads to higher molecular mass forms of  $\Delta F508$ -CFTR.** Because it is retained in the ER,  $\Delta F508$ -CFTR does not acquire a mature glycosylation pattern indicative of oligosaccharide modification in the Golgi apparatus (5). Wild-type CFTR, as expressed in COS-7 cells, is found as three differentially glycosylated species that can be distinguished by molecular mass: band A (molecular mass  $\sim 130$  kD) is unglycosylated, band B (molecular mass 150–170 kD) is core glycosylated in the ER, and band C (molecular mass 180–190 kD) has a mature glycosylation pattern characteristic of oligosaccharide processing in the Golgi apparatus. In COS-7 cells expressing  $\Delta F508$ -CFTR, only band B is observed (5).  $\Delta F508$ -CFTR in IB3-1 cells shows a similar pattern; there is typically a single immunoreactive band at molecular mass  $\sim 160$  kD consistent with band B (Fig. 2 A). In some experiments, a species of molecular mass  $\sim 140$  kD consistent with band A was observed.

We next tested whether 4PBA would induce  $\Delta F508$ -CFTR maturation consistent with that of wild-type CFTR's passage through the Golgi apparatus by examining CFTR immunoreactivity on protein immunoblots of IB3-1 cell lysates. In Fig. 2 A, IB3-1 cells were incubated for 3 d under control conditions at 37°C, in control media containing 0.1 mM 4PBA at 37°C, with 0.5 mM 4PBA at 37°C, or in control media at 27°C. Whole cell lysates were prepared and CFTR was detected by immunoblot analysis using a rabbit antiserum against a peptide NH<sub>2</sub>-terminal to nucleotide binding domain 1 (antiserum 181) (20) after SDS-PAGE. Band C was readily detected in the 0.5 mM 4PBA and the 27°C samples, less readily detected in the 0.1 mM 4PBA sample, and not in the control sample. Densitometric analysis of this experiment suggests that treatment of IB3-1

cells with 0.5 mM 4PBA results in approximately fourfold increased total immunoreactive CFTR protein, with approximately one-fourth of that immunoreactivity found as band C. For comparison, incubation at 27°C resulted in approximately fivefold increased immunoreactivity with again approximately one-fourth found as band C.

Fig. 2 B depicts the time course of the appearance of band C in IB3-1 cell lysates after treatment with 4PBA or the chemical chaperone glycerol. Total CFTR immunoreactivity, first in the form of band B, began to increase after 2–4 h of exposure to 4PBA or glycerol. Band C formation was noted at 6 (glycerol) to 8 h (4PBA). Band B and band C expression were both stable for at least 48 h. These kinetics are similar to those published for incubations of heterologous cells expressing  $\Delta F508$ -CFTR at reduced temperatures (12) or in the presence of the chemical chaperone glycerol (13, 14). In a parallel experiment, the concentration of 4PBA present in the growth media (measured by high performance liquid chromatography [26]) remained essentially constant at 1 mM over the same 48-h time course.

**Immunocytochemical detection of  $\Delta F508$ -CFTR in 4PBA-treated cells.** We next visualized  $\Delta F508$ -CFTR immunoreactivity after 4PBA treatment in IB3-1 cells. Cells were grown in the absence or presence of 1 mM 4PBA for 3 d at 37°C. Positive control conditions were IB3-1 cells grown in 1 M glycerol at 37°C or at 27°C. Immunocytochemistry was performed on fixed cells using a rabbit antiserum directed against a CFTR regulatory domain peptide (antiserum 169) (20). Representative data are shown in Fig. 3. Glycerol and 27°C increased CFTR immunostaining and were associated with morphologic changes. 1 mM 4PBA uniformly increased CFTR immunostaining without apparent morphologic changes. The effect of 4PBA was dose dependent. In preliminary experiments, cells treated with  $< 0.1$  mM 4PBA had no increase in CFTR immunoreactivity, while cells treated with 0.1, 0.5, 1, and 10 mM



**Figure 3.** Immunocytochemical detection of CFTR in IB3-1 cells. IB3-1 cells were fixed after 3 d of exposure to the indicated conditions. CFTR immunoreactivity was detected as described in Methods and appears as a blue stain. The counterstain is Nuclear Fast red. The bar represents 12  $\mu\text{m}$  for the control and glycerol panels, and 6  $\mu\text{m}$  for the PBA and 27°C panels.

4PBA had qualitatively increasing 4PBA immunoreactivity (data not shown). These data are consistent with 4PBA, increasing expression of  $\Delta\text{F508}$ -CFTR in a manner similar to that of 1 M glycerol and incubation at reduced temperature. These data are also consistent with our immunoblot data that 4PBA increases CFTR expression in IB3-1 cells.

When IB3-1 cells were grown in 10 mM butyrate, cell death occurred within 24 h, and morphologic changes and decreased growth rate were apparent at 1 mM butyrate. These concentrations of butyrate are lower than those tested in CFPAC cells (5–25 mM), a pancreatic acinar carcinoma cell line homozygous for  $\Delta\text{F508}$ -CFTR (10), suggesting that IB3-1 cells are more sensitive to toxic effects of butyrate than CFPAC cells.

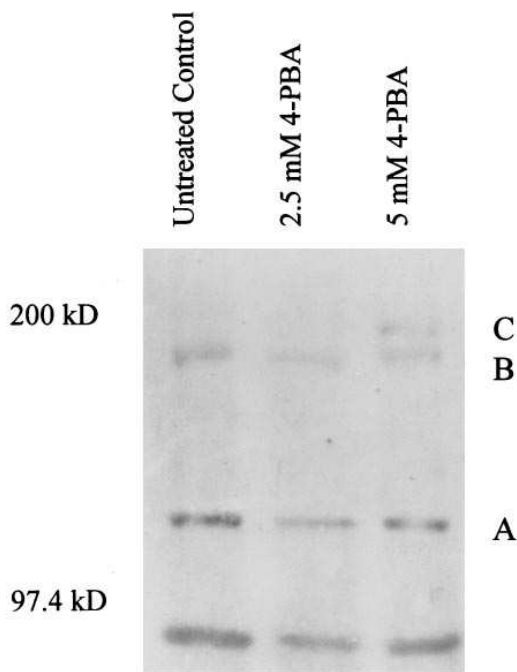
*Primary cultures of CF nasal epithelial cells also respond to 4PBA.* Nasal epithelial cells from CF patients in primary culture also respond to 4PBA treatment in vitro. Fig. 4 shows an immunoblot from an experiment similar to that shown for IB3-1 cells in Fig. 2. Primary cultures of nasal epithelial cells from a CF patient (genotype  $\Delta\text{F508}$ /unidentified mutation) were grown in control media in the absence or presence of 2.5 or 5 mM 4PBA for 6 d in culture at 37°C. Only bands A and B (molecular masses  $\sim 130$  and  $\sim 170$  kD) were observed for untreated cells and cells grown in the presence of 2.5 mM 4PBA. Band C ( $\sim 190$  kD), as well as bands A and B, was observed for cells grown in the presence of 5 mM 4PBA. Densitometry of this experiment suggests a 2.5-fold increase in immunoreactive CFTR protein (bands A + B + C) after treatment with

5 mM 4PBA, with band B having twice as much CFTR immunoreactivity as band C after treatment with 5 mM 4PBA.

Although higher concentrations of 4PBA are required to increase band C expression in the primary nasal epithelia than in IB3-1 cells, these results are similar to those seen in IB3-1 cells. Therefore, these data suggest that the 4PBA-induced maturation of  $\Delta\text{F508}$ -CFTR is neither an artifact of cell transformation nor an effect of 4PBA on the W1282X allele present in IB3-1 cells. While the identity of the second mutant CFTR allele in these primary nasal epithelial cells is unknown, we do know that it is not W1282X,  $\Delta\text{F508}$ , N1303K, G542X, G551D, or R553X. These alleles were part of this patient's screen during genotype determination. However, it remains unclear why higher concentrations of 4PBA were required to observe this effect in primary nasal epithelial cells than in immortalized IB3-1 cells.

4PBA treatment also resulted in an increase in CFTR immunoreactivity in primary nasal epithelial cells from a patient with genotype  $\Delta\text{F508}/\Delta\text{F508}$  (Fig. 5). The effect of 4PBA was again dose dependent; treatment of these cells with concentrations of 4PBA  $< 1$  mM showed little increase in CFTR immunoreactivity (data not shown). These data further suggest that 4PBA stimulates expression of  $\Delta\text{F508}$ -CFTR and not an alternative mutant allele present in IB3-1 cells or in the nasal epithelial cells assayed in Fig. 4.

*4PBA does not alter phenotype in cells expressing functional CFTR.* In a control experiment, we also assessed the influence



**Figure 4.** Immunoblot of cell lysates of 4PBA-treated primary nasal epithelium from a CF patient demonstrates molecular mass shifts consistent with oligosaccharide maturation. Nasal polyp epithelial cells from a CF patient with genotype  $\Delta F508$ /unknown were grown in primary culture with the indicated concentration of 4PBA for 6 d at 37°C. Cellular lysates were prepared by solubilization with 10% SDS at 95°C. 16  $\mu\text{g}$  of total protein was resolved on a 6% SDS-polyacrylamide gel before electrophoretic transfer to nitrocellulose for immunodetection of CFTR as previously described in the legend to Fig. 2.

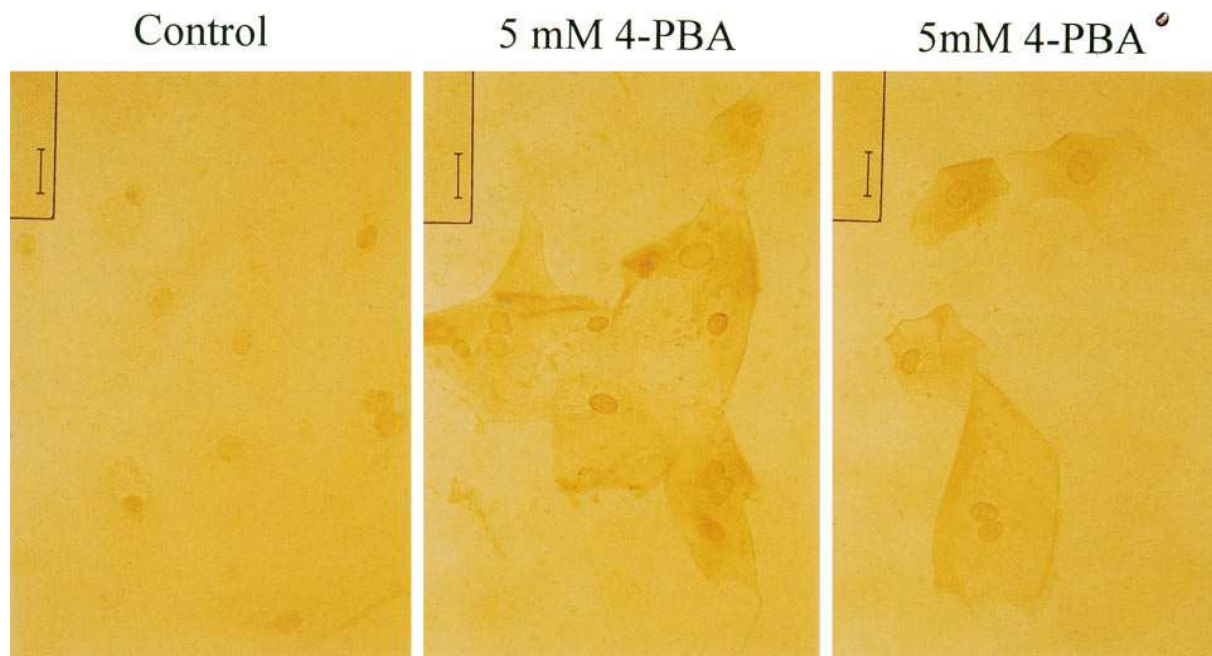
of 4PBA on IB3-1 cells that had been stably transfected with a functional CFTR allele (C38 cells) (27). Unlike the parent IB3-1 cells, C38 cells demonstrate a “rescued” phenotype, namely they demonstrate a stimulation of chloride secretion in response to forskolin, and CFTR-like channel activity on the plasma membrane. Incubation of C38 cells with 0.5 mM did not affect the rate of forskolin-stimulated chloride secretion; both control and 4PBA-treated C38 cells had an  $\sim 1.4$ -fold increase in rate of chloride efflux in response to forskolin. Thus, 4PBA did not alter the phenotype of  $\Delta F508$ -CFTR-containing cells in the presence of a functional CFTR allele. This magnitude of stimulation of chloride secretory rate in untreated C38 cells is similar to the parent IB3-1 cells after 4PBA treatment.

One possible explanation for not observing an additional effect of 4PBA in C38 cells is that the expression of a functional CFTR allele would cause downregulation of  $\Delta F508$ -CFTR expression, and therefore remove the target of 4PBA action. Another explanation is that the phenotypic presence of  $\Delta F508$ -CFTR is masked by presence of functional CFTR activity.

## Discussion

**Mechanism.** Collectively, these data suggest that an approved pharmaceutical agent, 4PBA, at clinically relevant concentrations (26), restores  $\Delta F508$ -CFTR to the normal pathway for trafficking to the plasma membrane. The experiments reported here do not distinguish between the possible mechanisms of 4PBA action.

4PBA was developed as a plasma ammonia scavenger for treatment of patients with urea cycle defects (26, 28). During early clinical trials of 4PBA, it was observed that circulating fe-



**Figure 5.** Immunocytochemical detection of CFTR in primary nasal epithelial cells from a CF patient. Primary nasal epithelial cells from a CF patient with genotype  $\Delta F508/\Delta F508$  were grown in growth media without 4PBA (control) or containing 5 mM 4PBA for 3 d at 37°C. Cells were fixed with 10% buffered formalin and permeabilized with 95% methanol before immunocytochemical detection of CFTR. The primary antiserum was a rabbit anti-human CFTR regulatory domain peptide antiserum (antiserum 169) and the secondary antibody was a donkey anti-rabbit IgG-horseradish peroxidase conjugate. Immunoreactivity was detected with Histomark orange (brown positive reaction product) without a counterstain as described in Methods. The bar represents 6  $\mu\text{m}$ .

tal hemoglobin increased in these patients (15). Therefore, subsequent clinical trials using 4PBA to raise fetal hemoglobin levels in patients with hemoglobinopathies were undertaken (29). 4PBA is also being used to promote differentiation of malignant cells *in vivo* and *in vitro* (30). The mechanism by which 4PBA raises circulating fetal hemoglobin and causes differentiation of tumor cells is different from its mechanism of action as a plasma ammonia scavenger and involves transcriptional upregulation of the relevant genes (30). Both 4PBA and its initial metabolite, phenylacetate (PAA), act to increase fetal hemoglobin levels by increasing transcription of  $\gamma$ -globin mRNA while decreasing transcription of  $\beta$ -globin mRNA (15). This action is similar to that of the transcriptional regulator butyrate (15).

Since 4PBA is an analogue of butyrate, a known transcriptional regulator of CFTR expression (10), it is reasonable to hypothesize that 4PBA increases transcription of the  $\Delta F508$ -CFTR allele. As a transcriptional regulator, 4PBA would increase levels of  $\Delta F508$ -CFTR protein, and by mass action, would force some  $\Delta F508$ -CFTR to bypass quality control in the ER.

This mechanism is consistent with the observations that butyrate itself can induce cAMP-responsive chloride secretion in a  $\Delta F508$ -homozygous pancreatic acinar cell line (10). Our data are also consistent with 4PBA increasing the amount of  $\Delta F508$ -CFTR protein produced; in the immunoblot experiments (Figs. 2 and 4) increased CFTR immunoreactivity was observed in the 4PBA-treated samples when equal amounts of cell lysate protein were assayed. Increased CFTR immunoreactivity was also observed by immunocytochemistry after 4PBA treatment (Figs. 3 and 5).

Confirmation of this mechanism of 4PBA action will require direct quantification of CFTR mRNA. CFTR mRNA is present at very low levels in the cells of the respiratory epithelia, typically below the level of detection by standard quantitative techniques such as Northern analysis and ribonuclease protection. Direct quantification of CFTR mRNA by either of these methods in untreated respiratory epithelial cells has not been published. The best estimates of CFTR mRNA copy number, determined by semiquantitative reverse-transcriptase polymerase chain reaction experiments, estimate that one to two copies of CFTR mRNA are present per cell (31). However, this method is insensitive to less than twofold differences in transcript level (31). We have attempted to directly measure CFTR mRNA in IB3-1 cells by ribonuclease protection using a probe that detects CFTR mRNA in kidney (32), and with two other probes constructed in our laboratory, but were unable to reproducibly detect CFTR mRNA after treatment with 1 mM 4PBA. This suggests that CFTR mRNA levels remain low in IB3-1 cells even after 4PBA treatment.

Butyrate and 4PBA have effects in IB3-1 cells that are qualitatively different from one another. IB3-1 cells treated with 1–2 mM 4PBA are healthy, grow at a similar rate and with a similar morphology to control cells, and express CFTR channel activity at the plasma membrane. Equimolar concentrations of butyrate cause morphologic changes in IB3-1 cells with rounding of cells and decreased growth rate. This suggests that 4PBA and butyrate may have different toxicity profiles and dose–response relationships. Our observations with 4PBA similarly differ from published observations with butyrate in  $\Delta F508$ -CFTR transfected C-127 cells; band C (the  $\sim 180$ -kD mature glycosylated species of CFTR) was not observed after

5 mM butyrate treatment for 24 h despite a massive increase in  $\Delta F508$ -CFTR mRNA as demonstrated by Northern analysis (10). In our experiments in IB3-1 cells, we were not able to assess the effect of butyrate treatment at millimolar concentrations because such treatments were associated with changes in cellular morphology and cell death. While 4PBA may act as a butyrate analogue to increase transcription of CFTR, the improvement with respect to toxicity at similar concentrations suggests that the mechanism of action of 4PBA may not be limited to increasing  $\Delta F508$ -CFTR transcription.

Our data in primary nasal epithelia also differ from those of Cheng et al. (10) with respect to primary nasal epithelia. 5 mM 4PBA led to formation of band C (Fig. 4) and increased immunoreactive CFTR protein (Fig. 5) in our experiments. Our cells were grown on solid supports, and we did not assess the electrolyte transport function of these cells. Cheng et al. (10) grew primary nasal epithelia from CF patients on semipermeable supports in Ussing chambers to generate a monolayer with tight junctions. In this system, they did not detect increases in cAMP-stimulated current indicative of chloride transport after treatment with up to 300 mM butyrate. There are a number of possible explanations for these differences in results. The simplest explanation is that 4PBA and butyrate may have overlapping, but not identical effects on nasal epithelial cells, as is suggested above. Demonstration of the effects of 4PBA on polarized epithelial monolayers is under investigation.

Our data are qualitatively similar to those obtained when cells expressing  $\Delta F508$ -CFTR are incubated with 1 M or 10% glycerol (Figs. 2 B and 3) (13, 14). Glycerol stabilizes thermally labile proteins during purification and storage, and may act by stabilizing an intrinsically thermolabile  $\Delta F508$ -CFTR such that a “partially unfolded” conformation is no longer recognized by the ER quality control mechanism. Glycerol has been shown by several groups to significantly improve processing and trafficking of  $\Delta F508$ -CFTR similar to growth at 25–27°C (12, 19).

Growth at reduced temperatures leads to thermal stabilization of cellular contents. The degree of thermal stabilization that is achieved by a 10°C temperature decrease is  $\sim 2$ –3 kcal/mol; 2–3 kcal/mol is approximately the energy of either one or two hydrogen bonds or a small number of hydrophobic interactions. Phenylalanine 508 resides in a region of predicted  $\beta$ -sheet secondary structure in nucleotide binding fold 1 of CFTR (33, 34). A peptide corresponding to CFTR nucleotide binding domain 1 (NBD-1, residues 404–589) is more readily denatured by guanidinium or increasing temperature when phenylalanine 508 is deleted (35). The mutant peptide is also more likely to form aggregates than the wild-type NBD-1 peptide (35). These observations are consistent with a thermodynamic destabilization of CFTR by the deletion of F508.

Glycerol is considered a member of the chemical chaperone class of compounds because of its thermal stabilization properties. Structurally, 4PBA and glycerol are not particularly similar. One can speculate that high concentrations of glycerol would increase the viscosity of the aqueous environment which would slow molecular motion and decrease the rate of denaturation. 4PBA might provide thermal stabilization by favoring the formation of a missing hydrogen bond or hydrophobic interaction. It also might directly disrupt the interaction of  $\Delta F508$ -CFTR and a molecular chaperone such as hsp70 (7) or calnexin (6). Alternatively, 4PBA might act as a transcriptional regulator of a protein involved in the ER quality control system. Alteration of expression of this hypotheti-

cal agent would influence the ability of the cell to block  $\Delta F508$ -CFTR trafficking.

It is also as yet unclear whether the active species of 4PBA is 4PBA, one of its metabolites, or both. As in the case for the urea cycle disorders where 4PBA is a prodrug and phenylacetate (the product of 4PBA  $\beta$ -oxidation) is the active species for scavenging ammonia (26), phenylacetate may be the active compound for normalizing  $\Delta F508$ -CFTR trafficking. Arguing against phenylacetate as the active compound is the lack of conversion of 4PBA to phenylacetate in the culture media of IB3-1 cells over a 2-d incubation.

**Therapy.** Manipulation of aberrant intracellular  $\Delta F508$ -CFTR trafficking has great promise as a pharmacologic treatment for the majority of CF patients. This approach, inducing the expression of a functional native protein by correcting its intracellular trafficking, circumvents many of the problems associated with other strategies for restoration of CFTR function, namely gene delivery systems and long-term persistence. The concentrations of 4PBA used in these experiments are similar to those routinely achieved in the plasma of patients who receive 4PBA therapy for urea cycle disorders (28). These factors make the use of 4PBA attractive as a potential pharmacologic therapy in CF patients who have at least one  $\Delta F508$ -CFTR allele.

**Conclusions.** 4PBA promotes escape of a fraction of  $\Delta F508$ -CFTR from the ER and its appearance at the plasma membrane in cells derived from the respiratory epithelia of CF patients. This cell surface  $\Delta F508$ -CFTR exists as linear, 10 pS chloride channel activated by PKA and ATP. The mechanism by which 4PBA acts is unclear, but preliminary data suggest that 4PBA may have overlapping but different overall effects than butyric acid, a known regulator of CFTR gene expression. Clinical trials will be required to determine the safety and utility of 4PBA as a therapy for CF patients.

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