PBA increases CFTR expression but at high doses inhibits Cl− secretion in Calu-3 airway epithelial cells

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SODIUM 4-PHENYLBUTYRATE (PBA), a short-chain fatty acid that functions as an ammonia scavenger, has recently been approved by the Food and Drug Administration to treat patients with urea cycle enzyme deficiencies (29). Because PBA increases fetal hemoglobin levels, it is also being evaluated in the management of sickle cell disease and thalassemia (6). PBA also causes cellular differentiation and is used in phase I clinical trials as a tumor-differentiating agent (12, 28, 29). PBA and its analog sodium butyrate have numerous and diverse cellular effects. These include modulation of protein kinase, phosphatase, and ecto-5’-nucleotidase activities (7, 28, 30); stimulation of microtubule and microfilament formation (1); inhibition of colonic Cl− secretion (8); induction of apoptosis (12); and transcriptional activation of numerous genes, including heat shock proteins (12) and the cystic fibrosis transmembrane conductance regulator (CFTR) (32, 33).

PBA is under evaluation as a treatment for cystic fibrosis (CF), a systemic disease caused by mutations in the CFTR gene encoding a CAMP-activated Cl− channel (25, 32, 33). Seventy percent of individuals with CF express ΔF508 CFTR, a mutation that prevents the export of CFTR from the endoplasmic reticulum to the apical plasma membrane, resulting in the inability of CAMP to stimulate Cl− secretion across epithelial cells in the respiratory tract and pancreas (5, 11, 37, 37). Overexpression of ΔF508 CFTR, a reduction in temperature, and chemical chaperones allow some ΔF508 CFTR export out of the endoplasmic reticulum to the plasma membrane, where ΔF508 CFTR functions as a CAMP-activated Cl− channel (9, 22, 34). PBA increases ΔF508 CFTR expression and restores CAMP-activated Cl− secretion in CF nasal airway epithelial and CF bronchial epithelial cells (32, 33). Thus PBA could be a useful therapy for CF in patients expressing ΔF508 CFTR.

Because little is known about the effects of PBA on wild-type (wt) CFTR expression and function in epithelial cells, the goal of this study was to examine the effects of PBA on wt CFTR-mediated Cl− secretion. To this end, we studied Calu-3 cells, a human airway epithelial cell line that expresses endogenous wt CFTR and has a serious cell phenotype. We report that chronic treatment of Calu-3 cells with a high concentration (5 mM) of PBA, sodium butyrate, or sodium valproate but not sodium acetate reduced basal and 8-(4-chlorophenylthio)-cAMP-stimulated Cl− secretion. Paradoxically, PBA enhanced CFTR protein expression 6- to 10-fold and increased the intensity of CFTR staining in the apical plasma membrane. PBA also increased CFTR expression 6- to 10-fold and increased the intensity of CFTR staining in the apical plasma membrane. PBA increases CFTR expression but at high doses inhibits Cl− secretion across Calu-3 cells. We conclude that PBA concentrations in the therapeutic range are unlikely to have a negative effect on Cl− secretion. However, concentrations >5 mM might reduce transepithelial Cl− secretion by serous cells in submucosal glands in individuals expressing wt CFTR.

To examine the effects of PBA on wt CFTR expression and function in wt airway epithelial cells, we studied Calu-3 cells, a human airway cell line with a serous cell phenotype (13, 15, 26, 35, 36). Serous cells in human airway submucosal glands secrete Cl− via CFTR located in the apical cell membrane and secrete antibiotic-rich fluid (10). 8-(4-Chlorophenylthio)-cAMP (CPT-cAMP)-stimulated Cl− secretion across Calu-3 cells is a two-step process: uptake across the basolateral membrane is mediated primarily by a Cl−/HCO3− exchanger, although an Na+−K+−2Cl−cotransporter could also nominally contribute to Cl− uptake, and secretion across the apical membrane is mediated by CFTR (13, 15, 20, 26, 35, 36). We report that chronic treatment of Calu-3 cells with a high concentration of PBA (5 mM) reduced basal and CPT-cAMP-stimulated Cl− secretion. Paradoxically, PBA enhanced CFTR protein expression 6- to 10-fold and increased the intensity of CFTR staining in the apical plasma membrane. PBA also increased protein expression of Na+−K+−ATPase and Na+−K+−2Cl−/HCO3− exchanger, resulting in the inability of CAMP to stimulate Cl− secretion across epithelial cells in the respiratory tract and pancreas (5, 11, 37, 37).

Because little is known about the effects of PBA on wild-type CFTR expression and function in epithelial cells, the goal of this study was to examine the effects of PBA on wt CFTR-mediated Cl− secretion in airway epithelial cells. To this end, we studied Calu-3 cells, a human airway cell line with a serous cell phenotype (13, 15, 26, 35, 36). Serous cells in human airway submucosal glands secrete Cl− via CFTR located in the apical cell membrane and secrete antibiotic-rich fluid (10). 8-(4-Chlorophenylthio)-cAMP (CPT-cAMP)-stimulated Cl− secretion across Calu-3 cells is a two-step process: uptake across the basolateral membrane is mediated primarily by a Cl−/HCO3− exchanger, although an Na+−K+−2Cl−cotransporter could also nominally contribute to Cl− uptake, and secretion across the apical membrane is mediated by CFTR (13, 15, 20, 26, 35, 36). We report that chronic treatment of Calu-3 cells with a high concentration of PBA (5 mM) reduced basal and CPT-cAMP-stimulated Cl− secretion. Paradoxically, PBA enhanced CFTR protein expression 6- to 10-fold and increased the intensity of CFTR staining in the apical plasma membrane. PBA also increased protein expression of Na+−K+−ATPase. However, PBA reduced CFTR-mediated Cl− currents across the apical membrane and had no effect on Na+−K+−ATPase activity in the basolateral membrane. Thus high concentrations of PBA (5 mM) inhibit Cl− secretion across

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Calu-3 cells primarily by inhibiting CFTR Cl\(^-\) currents across the apical membrane. In contrast, lower therapeuetic concentrations of PBA had no effect on cAMP-stimulated Cl\(^-\) secretion across Calu-3 cells.

**METHODS**

Cell culture. Calu-3 cells were obtained at passage 17 from the American Type Culture Collection (Manassas, VA) and cultured in tissue culture flasks (Costar, Cambridge, MA) coated with Vitrogen plating medium (VPM) containing DMEM (JRH Biosciences, Lenexa, KS), human fibronectin (10 µg/ml; Collaborative Biomedical Products, Bedford, MA), 1% Vitrogen-100 (Collagen, Palo Alto, CA), and BSA (10 µg/ml; Sigma Chemical, St. Louis, MO). Cells were then placed in an incubator maintained at 37°C and gassed with 5% CO\(_2\)-air. Every 48 h, 90% of the medium, MEM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FS; HyClone, Logan, UT), 2 mM L-glutamine (GIBCO BRL), 1 mM pyruvate, 50 U/ml penicillin, and 50 µg/ml streptomycin (Sigma), was replaced. At 90% confluence, cells were subcultured by incubation in Hanks' balanced salt solution containing trypsin (0.05%) and EDTA (0.53 mM; GIBCO BRL) and reseeded at a 1:7 dilution in VPM-coated cell culture flasks or plated at 1 × 10\(^6\) cells/0.6 cm\(^2\) on VPM-coated Millicell polycarbonate filters and grown in air-liquid interface culture (21, 26, 35). The medium was completely changed every 24 h when cells were grown on Millipore filters. Cells were studied 10-15 days after being seeded. Twenty-four hours before Ussing chamber experiments, FBS was removed from the cell culture medium. Vehicle, sodium butyrate (Sigma), PBA (Aldrich, Milwaukee, WI), valproic acid (Aldrich), or sodium acetate (Sigma) was added to the cell culture medium, as described (4, 8, 24, 32), from a filtered sterilized stock solution prepared immediately before use every 1, 2, 4, or 6 days (as indicated in RESULTS).

Measurement of short-circuit current. Short-circuit current (I\(_{sc}\)) was measured across monolayers of Calu-3 cells in the presence of amiloride (10\(^{-5}\) M) in the apical solution to block the potential contribution of Na\(^+\) transport to I\(_{sc}\) (16, 17, 27). In the presence of amiloride, CPT-cAMP-stimulated I\(_{sc}\) in Calu-3 cells is equivalent to electrogenic Cl\(^-\) secretion (21). Bath solutions were maintained at 37°C and were stirred by bubbling with 5% CO\(_2\)-air. Current output from the voltage clamp was digitized by a TL-1 DMA interface analog-to-digital converter (Axon Instruments, Foster City, CA). Data collection and analysis were done with Axotape 2.0 software (Axon Instruments). During experiments, cells were bathed in an FBS-free MEM solution containing (in mM) 116 NaCl, 24 NaHCO\(_3\), 3 KCl, 2 MgCl\(_2\), 0.5 CaCl\(_2\), 3.6 sodium HEPES, 4.4 hydrogen HEPES (pH 7.4), and 10 glucose.

To examine the effect of butyrate on the activity of the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, sections were preincubated with 1% SDS for 10 min at room temperature and then incubated with monoclonal antibody T4 (1:2,000), generously provided by Dr. B. Forbush (23), followed by a 1:25 dilution of a goat anti-mouse F(ab')\(_2\) fragment IgG-FITC (DAKO). To detect the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, sections were preincubated with 1% SDS for 10 min at room temperature and then incubated with monoclonal antibody T4 (1:2,000), generously provided by Dr. B. Forbush (23), followed by a 1:25 dilution of a goat anti-mouse F(ab')\(_2\) fragment IgG-FITC (DAKO). To identify cell nuclei, nucleic acids were stained with propidium iodide (2.5 µg/ml). Sections were mounted in DAKO-Glycergel (DAKO, Carpinteria, CA) containing 2.5% 1,4-diazabicyclo(2.2.2)octane to retard fading. Acute exposure to PBA (40 min) had no effect on the immunocytochemical localization of CFTR, Na\(^+\)-K\(^+\)-ATPase, or the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter compared with untreated cells. Moreover, in cells treated with PBA for 6 days, removal of PBA from the cell culture medium 2 h before processing did not alter the immunolocalization of the transporters compared with cells treated continuously with PBA for 6 days.

Fluorescent images were acquired by using a Zeiss confocal laser-scanning microscope (LSM 310; Carl Zeiss, Oberko chen, Germany) equipped with a ×63 PlanApochromat/1.4-NA oil-immersion objective. FITC fluorescence was excited with the use of the 488-nm argon laser line and collected by using a 515- to 570-nm band-pass filter. Propidium iodide fluorescence was excited by using the 543-nm helium-neon laser line and collected with the use of a 575-77-nm long-pass filter. All images were acquired by using the same confocal parameters and were imported into Adobe Photoshop version 3.0 for processing and printing.

Western blot analysis. Cell monolayers were solubilized in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% NP-40), containing the complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) for 60 min at 4°C, scraped from filters, and spun at 14,000 g for 4 min to pellet insoluble material. Supernatants were separated on 4-15% Tris-HCl gradient gels (Bio-Rad) and transferred to polyvinylidene difluoride Immobilon membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C in 5% nonfat dry milk in Tris-buffered saline-0.1% Tween 20 and incubated with either the CFTR carboxy-terminal monoclonal antibody...
PBA, sodium butyrate, and sodium valproate inhibit Cl⁻ secretion. As we previously demonstrated (21), CPT-cAMP rapidly increased I_{sc}, which reached a peak in 2 min and then declined to a steady-state value significantly above basal I_{sc} (Fig. 1). As illustrated in Figs. 1-4, PBA and sodium butyrate decreased basal and CPT-cAMP-stimulated I_{sc} after 1, 2, and 6 days of continuous treatment. In contrast, acute exposure to PBA or sodium butyrate (10 min before addition of CPT-cAMP) had no effect on basal or CPT-cAMP-stimulated Cl⁻ secretion (compare Figs. 2–4 with Fig. 6). In contrast, continuous exposure to sodium acetate (5 mM) for 2 days had no effect on basal or CPT-cAMP-stimulated Cl⁻ secretion by Calu-3 cells (n = 6 monolayers/group). However, at 5 mM, sodium valproate (2 days) inhibited basal and CPT-cAMP-stimulated I_{sc} (Fig. 6). At a comparable concentration (5 mM), sodium valproate elicited a larger inhibition of basal and CPT-cAMP-stimulated I_{sc} than sodium butyrate (compare Figs. 2–4 with Fig. 6). In contrast, continuous exposure to sodium acetate (5 mM) for 2 days had no effect on basal or CPT-cAMP-stimulated I_{sc} (Fig. 7).

Dose-response studies: PBA and Cl⁻ secretion. Ruben-stein and Zeitlin (33) report that a therapeutic effect of PBA was observed in CF patients when plasma concentrations of PBA were between 0.05 and 2 mM. To determine whether therapeutic concentrations of PBA inhibit Cl⁻ secretion across Calu-3 cells, we examined
the effect of PBA at concentrations between 0.05 and 2 mM for 4 days. As shown in Fig. 8, concentrations of PBA between 0.05 and 2 mM had no effect on CPT-cAMP-stimulated Cl\textsuperscript{2} secretion. However, as reported above, 5 mM PBA reduced CPT-cAMP-stimulated Cl\textsuperscript{2} secretion (Fig. 8). Thus therapeutic concentrations of PBA have no effect on CPT-cAMP-stimulated Cl\textsuperscript{2} secretion across Calu-3 cells.

Effect of PBA on CFTR, Na\textsuperscript{1}-K\textsuperscript{1}-ATPase, and Na\textsuperscript{1}-K\textsuperscript{1}-2Cl\textsuperscript{2} expression. We examined the possibility that PBA (5 mM) might decrease CFTR-mediated Cl\textsuperscript{2} secretion by altering the expression of key transport proteins involved in transepithelial Cl\textsuperscript{2} secretion in Calu-3 cells, including CFTR, Na\textsuperscript{1}-K\textsuperscript{1}-ATPase, and the Na\textsuperscript{1}-K\textsuperscript{1}-2Cl\textsuperscript{2} cotransporter (35). CFTR mediates Cl\textsuperscript{2} secretion across the apical cell membrane, and Na\textsuperscript{1}-K\textsuperscript{1}-ATPase maintains a low intracellular concentration of Na\textsuperscript{+}, which is important for providing the driving force for Cl\textsuperscript{2} entry across the basolateral membrane primarily by Cl\textsuperscript{2}/HCO\textsubscript{3}\textsuperscript{2} exchange. However, the Na\textsuperscript{1}-K\textsuperscript{1}-2Cl\textsuperscript{2} cotransporter also contributes to Cl\textsuperscript{2} uptake (35). Operation of the Cl\textsuperscript{2}/HCO\textsubscript{3}\textsuperscript{2} exchanger and the Na\textsuperscript{1}-K\textsuperscript{1}-2Cl\textsuperscript{2} transporter maintains Cl\textsuperscript{2} above the apical membrane electrochemical equilibrium potential, thereby establishing the driving force for Cl\textsuperscript{2} secretion across the apical membrane into the airway surface fluid (35). Western blot analysis revealed that PBA and sodium butyrate increased CFTR and Na\textsuperscript{1}-K\textsuperscript{1}-ATPase protein levels (Figs. 9 and 10). The increase in transport protein expression was surprising, given the observed decreased in Cl\textsuperscript{2} secretion. In contrast, PBA decreased Na\textsuperscript{1}-K\textsuperscript{1}-2Cl\textsuperscript{2} cotransporter expression (Figs. 9 and 10).

To provide additional support for the observation that sodium butyrate and PBA altered the expression

![Fig. 4. Effect of sodium butyrate and PBA on steady-state I\textsubscript{sc}. In this and subsequent figures, steady-state I\textsubscript{sc} is defined as current measured 10–12 min after decline of I\textsubscript{sc} from peak value after addition of CPT-cAMP (100 µM) to apical and basolateral bath solutions. *P < 0.05 vs. control.](image1)

![Fig. 5. Effect of acute exposure to sodium butyrate and PBA on I\textsubscript{sc}. Sodium butyrate (5 mM) or PBA (5 mM) was added to apical and basolateral bath solutions 10 min before basal I\textsubscript{sc} was measured. Hatched bars, control data (NaCl, 5 mM; n = 10); open bars, PBA data (n = 10); gray bars, sodium butyrate data (n = 10). SS, steady state.](image2)

![Fig. 6. Effect of sodium valproate on basal and CPT-cAMP-stimulated I\textsubscript{sc}. Hatched bars, control (vehicle, 0.1% ethanol, for 2 days; n = 4); open bars, sodium valproate (5 mM, for 2 days; n = 4). *P < 0.0001 vs. control.](image3)

![Fig. 7. Effect of sodium acetate on basal and CPT-cAMP-stimulated I\textsubscript{sc}. Hatched bars, control (vehicle, distilled water; n = 4); open bars, sodium acetate (5 mM, for 2 days; n = 4).](image4)
of CFTR, Na\(^{+}\)-K\(^{+}\)-ATPase, and the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter, we performed immunofluorescence microscopy studies. As described previously (21), CFTR was found in the apical plasma membrane of Calu-3 cells (Fig. 11, A and B). The vast majority of control cells expressed undetectable or very low levels of CFTR (Fig. 11A). Treatment with PBA (5 mM for 6 days) increased the intensity of CFTR immunostaining in the apical plasma membrane (Fig. 11, compare A with B). PBA also increased the intensity of Na\(^{+}\)-K\(^{+}\)-ATPase staining, which was localized predominantly in the lateral membrane in control and PBA-treated monolayers (Fig. 11, C and D). Thus PBA increased the amount of CFTR in the apical membrane and Na\(^{+}\)-K\(^{+}\)-ATPase in the basolateral membrane. In contrast, PBA decreased the intensity of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) staining in the basolateral membrane (Fig. 11, compare E with F). These immunofluorescence microscopy studies are consistent with our Western blot analyses, demonstrating that PBA increased CFTR and Na\(^{+}\)-K\(^{+}\)-ATPase expression but decreased Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter expression.

Effect of PBA on Na\(^{+}\)-K\(^{+}\)-ATPase activity and Cl\(^{-}\)-channel currents. To examine the effect of PBA on the activity of Na\(^{+}\)-K\(^{+}\)-ATPase, we permeabilized the apical membrane with nystatin and measured \(I_{sc}\), which, under these conditions, represents the activity of Na\(^{+}\)-K\(^{+}\)-ATPase (31). PBA had no effect on Na\(^{+}\)-K\(^{+}\)-ATPase activity. \(I_{sc}\) was 106.1 ± 3.5 µA/cm\(^2\) (n = 4) in control monolayers and 119.0 ± 6.9 µA/cm\(^2\) in monolayers exposed to PBA (5 mM for 2 days; n = 4, P = 0.20). Thus, although PBA increased Na\(^{+}\)-K\(^{+}\)-ATPase protein expression, the drug had no effect on Na\(^{+}\)-K\(^{+}\)-ATPase activity. To examine the effect of PBA on apical membrane CFTR Cl\(^{-}\}-currents, we permeabilized the basolateral membrane with nystatin and measured \(I_{sc}\) in the presence of a transepithelial Cl\(^{-}\} concentration gradient directed from the apical to the basolateral solution. PBA dramatically reduced the \(I_{sc}\) flowing across apical
expression, the drug reduced the CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−}. Thus, although PBA increased CFTR protein concentrations of PBA (0.05–2 mM) had no effect on cAMP-treated with PBA (5 mM PBA for 2 days; n = 6, P < 0.01). Thus, although PBA increased CFTR protein expression, the drug reduced the CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−} currents. Taken together, the Western blot, confocal microscopy, and Ussing chamber studies suggest that PBA reduces CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−} secretion in part by inhibiting the activity of CFTR Cl\textsuperscript{−}\textsuperscript{−} channels in the apical membrane.

**DISCUSSION**

The major new finding of this report is that chronic treatment with the short-chain fatty acids PBA, sodium butyrate, and sodium valproate but not sodium acetate at a concentration of 5 mM reduced basal and CPT-CAMP-stimulated Cl\textsuperscript{−}\textsuperscript{−} secretion across polarized human airway epithelial cells (Calu-3) expressing wt CFTR. Paradoxically, PBA and sodium butyrate increased the expression of CFTR and Na\textsuperscript{+}-K\textsuperscript{−}-ATPase. However, PBA reduced CFTR Cl\textsuperscript{−}\textsuperscript{−} currents across the apical membrane. In contrast, lower therapeutic concentrations of PBA (0.05–2 mM) had no effect on CAMP-stimulated Cl\textsuperscript{−}\textsuperscript{−} secretion across Calu-3 cells. We conclude that PBA concentrations in the therapeutic range are unlikely to have a negative effect on Cl\textsuperscript{−}\textsuperscript{−} secretion. However, concentrations >5 mM might reduce transepithelial Cl\textsuperscript{−}\textsuperscript{−} secretion by serous cells in submucosal glands in individuals expressing wt CFTR.

Sodium butyrate also inhibits electrogenic Cl\textsuperscript{−}\textsuperscript{−} secretion across intestinal cell lines. In T-84 cells, a human colonic epithelial cell line, chronic sodium butyrate treatment (i.e., days) decreased cAMP-stimulated Cl\textsuperscript{−}\textsuperscript{−} secretion (18, 24). The inhibition of Cl\textsuperscript{−}\textsuperscript{−} secretion by sodium butyrate was mediated by a reduction of Na\textsuperscript{+}-K\textsuperscript{−}-2Cl\textsuperscript{−}\textsuperscript{−} cotransporter expression. Efflux through apical membrane CFTR Cl\textsuperscript{−}\textsuperscript{−} channels was not affected by sodium butyrate (24). In contrast, chronic sodium butyrate treatment had no effect on cAMP-activated Cl\textsuperscript{−}\textsuperscript{−} efflux across HT-29 cells, a human colonic epithelial cell line (24). Acute exposure (i.e., minutes) to sodium butyrate inhibited CAMP-stimulated Cl\textsuperscript{−}\textsuperscript{−} secretion by rat colon (8) and T-84 cells by reducing apical membrane Cl\textsuperscript{−}\textsuperscript{−} conductance (8). In contrast, in the present study, acute treatment with sodium butyrate had no effect on cAMP-stimulated Cl\textsuperscript{−}\textsuperscript{−} secretion. Although the reason(s) for these divergent observations is not clear, it is possible that the effects of sodium butyrate might be tissue specific as well as time and dose dependent.

Inhibition of CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−} secretion across Calu-3 cells by PBA and sodium butyrate was not observed at clinically relevant concentrations (i.e., 0.05–2 mM (29, 33)). Thus it is unlikely that PBA given to individuals with urea cycle enzyme deficiencies, sickle cell disease, thalassemia, or cancer would have the untoward effect of inhibiting Cl\textsuperscript{−}\textsuperscript{−} secretion across epithelial cells expressing wt CFTR. Sodium valproate at clinically relevant concentrations (i.e., 100 µM to 1 mM for 2 days) also had no effect on basal or CPT-cAMP-stimulated Cl\textsuperscript{−}\textsuperscript{−} secretion by Calu-3 cells. Inhibition of Cl\textsuperscript{−}\textsuperscript{−} secretion by sodium valproate was observed only at 5 mM. Thus it is unlikely that valproic acid therapy would alter Cl\textsuperscript{−}\textsuperscript{−} secretion in vivo in epithelial cells expressing wt CFTR.

In this report, we began to examine some of the possible mechanisms whereby PBA (5 mM) inhibits CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−} secretion across Calu-3 cells. We examined the possibility that PBA reduced Cl\textsuperscript{−}\textsuperscript{−} secretion by downregulating the expression of transport proteins involved in Cl\textsuperscript{−}\textsuperscript{−} secretion across Calu-3 cells, including CFTR, Na\textsuperscript{+}-K\textsuperscript{−}-ATPase, and the Na\textsuperscript{+}-K\textsuperscript{−}-2Cl\textsuperscript{−}\textsuperscript{−} cotransporter (19, 24, 36). Paradoxically, we found that PBA increased CFTR and Na\textsuperscript{+}-K\textsuperscript{−}-ATPase protein expression, effects that would be expected to stimulate, not inhibit, Cl\textsuperscript{−}\textsuperscript{−} secretion. However, our Ussing chamber studies revealed that PBA reduced CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−} currents across the apical membrane but had no effect on the activity of Na\textsuperscript{+}-K\textsuperscript{−}-ATPase. Thus the major mechanism whereby PBA reduces Cl\textsuperscript{−}\textsuperscript{−} secretion involves inhibition of apical CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−} currents, even though there are more CFTR Cl\textsuperscript{−}\textsuperscript{−} channels in the apical membrane. This later observation suggests that PBA could have dual effects on CFTR: 1) to increase the number of channels in the membrane and 2) to reduce the current flow through CFTR Cl\textsuperscript{−}\textsuperscript{−} channels. The mechanism of this later effect is unknown and requires additional studies.

It also must be considered that a reduction in Na\textsuperscript{+}-K\textsuperscript{−}-2Cl\textsuperscript{−}\textsuperscript{−} cotransporter and/or Cl\textsuperscript{−}\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange expression in the basolateral membrane might also contribute to the reduction in Cl\textsuperscript{−}\textsuperscript{−} secretion observed in PBA-treated cells. Although we report that PBA reduced Na\textsuperscript{+}-K\textsuperscript{−}-2Cl\textsuperscript{−}\textsuperscript{−} expression, this transporter contributes only nominally to CPT-cAMP-stimulated Cl\textsuperscript{−}\textsuperscript{−} secretion by Calu-3 cells (13, 15, 20, 26, 35, 36). Thus it is unlikely that the reduction in Na\textsuperscript{+}-K\textsuperscript{−}-2Cl\textsuperscript{−}\textsuperscript{−} cotransporter protein expression plays a major role in the inhibition by PBA of Cl\textsuperscript{−}\textsuperscript{−} secretion. Additional studies, including extensive investigation to define more completely the roles of the Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger and the Na\textsuperscript{+}-K\textsuperscript{−}-2Cl\textsuperscript{−}\textsuperscript{−} cotransporter, are required to elucidate the mechanism whereby PBA inhibits CFTR-mediated Cl\textsuperscript{−}\textsuperscript{−} secretion.

In conclusion, the observation that PBA (5 mM) has a negative effect on wt CFTR-mediated, cAMP-stimu-
Fig. 11. Effects of PBA on expression and localization of CFTR, Na\(^{+}\)-K\(^{+}\)-ATPase, and Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter. 

A: immunolocalization of CFTR in control cells. B: immunolocalization of CFTR in cells treated with PBA (5 mM) for 6 days. A and B: CFTR is red, and nuclei, stained with propidium iodide, are purple. PBA increased intensity of CFTR staining in apical plasma membrane and number of cells expressing CFTR. 

C: immunolocalization of Na\(^{+}\)-K\(^{+}\)-ATPase in control cells. D: immunolocalization of Na\(^{+}\)-K\(^{+}\)-ATPase in cells treated with PBA (5 mM) for 6 days. C and D: Na\(^{+}\)-K\(^{+}\)-ATPase is green, and nuclei are purple. PBA increased intensity of Na\(^{+}\)-K\(^{+}\)-ATPase staining in lateral membrane. 

E: immunolocalization of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter in control cells. F: immunolocalization of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter in cells treated with PBA (5 mM) for 6 days. E and F: Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter is green, and nuclei, stained with propidium iodide, are purple. PBA decreased intensity of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter staining in basolateral plasma membrane. 

For each transport protein examined, images in control and PBA-treated cells were obtained by using identical settings on confocal microscope and were printed with the use of same settings. It is important to note that Calu-3 cells maintained a polarized epithelium, as revealed in these images, capable of secreting Cl\(^{-}\) in a regulated manner (i.e., CPT-cAMP) even when exposed to a relatively high concentration of PBA (5 mM). Scale bar = 10 µm.
lated Cl− secretion in Calu-3 cells, a model cell type for human serous cells in submucosal glands in the trachea, does not appear to have implications for the clinical use of this drug for patients with CF, urea cycle enzyme deficiencies, sickle cell disease, thalassemia, and cancer as long as the therapeutic concentrations are in the range of 0.05–2 mM. However, we conclude that plasma PBA levels of 5 mM or above in individuals expressing wt CFTR could have untoward effects on transepithelial Cl− secretion by serous cells in the submucosal glands.

We thank Dr. Jack McBain for helpful suggestions; Dr. David McCoy and Collin Shaw for performing some of the Ussing chamber experiments; Kerry O'Brien, Melissa Levak, Lea Klauśli, and Jerry Denton for valuable technical assistance; and Dr. Alice Givan and Ken Orndorff for assistance with confocal microscopy. We also thank the anonymous reviewers for helpful comments on the manuscript and for the suggestion to conduct dose-response studies with sodium 4-phenylbutyrate.

These studies were supported by grants from the National Institutes of Health (DK/HL-45881) and the Cystic Fibrosis Foundation (to B. A. Stanton). Loffing was supported by a fellowship from the Dolores Zohrab Liebmann Foundation. Address for reprint requests and other correspondence: B. A. Stanton, Dept. of Physiology, 615 Remsen Bldg., Dartmouth Medical School, Hanover, NH 03755 (E-mail: Bruce.A.Stanton@Dartmouth.edu).

Received 21 October 1998; accepted in final form 17 May 1999.

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