Sodium 4-phenylbutyrate (4-PBA) has been shown to correct the cellular trafficking of several mutant or nonmutant plasma membrane proteins such as cystic fibrosis transmembrane conductance regulator through the expression of 70-kDa heat shock proteins. The objective of the study was to determine whether 4-PBA may influence the functional expression of epithelial sodium channels (ENaC) in human nasal epithelial cells (HNEC). Using primary cultures of HNEC, we demonstrate that 4-PBA (5 mM for 6 h) markedly stimulated amiloride-sensitive sodium transport and in functional expression of ENaC as demonstrated by the increase in ENaC cell surface expression (i) was due to insertion of newly ENaC subunits as determined by brefeldin A experiments and (ii) was not associated with cell surface retention of ENaC subunits because endocytosis of ENaC subunits was unchanged. In addition, we find that ENaC co-immunoprecipitated with the heat shock protein constitutively expressed Hsc70, that has been reported to modulate ENaC trafficking, and that 4-PBA decreased Hsc70 protein level. Finally, we report that in cystic fibrosis HNEC obtained from two cystic fibrosis patients, 4-PBA increased functional expression of ENaC as demonstrated by the increase in amiloride-sensitive sodium transport and in α-, β-, and γ-ENaC subunit expression in the apical membrane. Our results suggest that in HNEC, 4-PBA increases the functional expression of ENaC through the insertion of new α-, β-, and γ-ENaC subunits into the apical membrane and also suggest that 4-PBA could modify ENaC trafficking by reducing Hsc70 protein expression.

Efficient clearance of mucus and inhaled pathogens from the respiratory tract is dependent on an optimal airway surface liquid (ASL), the volume of which is tightly regulated by active sodium and chloride transports across the airway epithelium with water following passively (1). In airway epithelial cells, sodium enters the apical membrane mainly through the epithelial sodium channel (ENaC). ENaC and the cystic fibrosis transmembrane conductance regulator (CFTR) are co-localized at the apical surface of respiratory epithelia where CFTR can regulate ENaC activity (2, 3). The activity of ENaC appears increased in cystic fibrosis (CF) compared with non-CF airway epithelia, which likely leads to enhanced absorption of water from the ASL (4). The role of ENaC in ASL volume regulation was recently confirmed by Mall et al. (5), who showed that airway-targeted overexpression of β-ENaC in mouse airways increased in vivo sodium absorption, caused ASL volume depletion, and initiated a cystic fibrosis-like lung disease. Thus, the regulation of ENaC expression and activity is likely to be critical in airway homeostasis.

Relatively little is known about the regulation of ENaC processing, trafficking, and stability at the cell surface of airway epithelial cells under physiological or pathological conditions. Recent studies indicate that ENaC intracellular trafficking shares some similarities with CFTR maturation: both channels are processed inefficiently with only a small fraction of newly synthesized proteins reaching the membrane, and the major fraction is targeted for intracellular degradation by the ubiquitin proteasome system (6). Such degradation depends in part on the constitutively expressed 70-kDa heat shock protein (Hsc70), which is an essential factor for ubiquitination (7). Sodium 4-phenylbutyrate (4-PBA) has been shown to function as a chemical chaperone that can correct the cellular trafficking of misfolded mutant AF508-CFTR plasma proteins leading to increased cell surface expression and enhanced chloride flux (8). Also, 4-PBA was shown to increase cell surface expression of non mutant proteins such as Na,K-ATPase, but paradoxi-
cally 4-PBA did not change or even decreased Na,K-ATPase activity in epithelial cell lines (9). The mechanisms by which 4-PBA elicits proper maturation of mutant and non mutant proteins remain unclear. Several studies have reported that 4-PBA reduced the expression of Hsc70 in CF epithelial cells (10), leading to a decrease in Hsc70-ΔF508 CFTR association and therefore to a decrease in ΔF508 degradation via the ubiquitin-proteasome system (7). To date, the effect of 4-PBA on the expression and function of ENaC in native human epithelial cells has not been evaluated.

The objectives of the present study were to determine whether 4-PBA can modulate ENaC cell surface expression and activity in human nasal epithelial cells (HNEC) and to evaluate the mechanisms involved in this effect. The results indicate that 4-PBA increased apical cell surface expression of ENaC and concomitantly enhanced amiloride-sensitive sodium transport in HNEC. 4-PBA-induced increase in ENaC expression in the apical membrane was due rather to insertion of new sodium channels from the cellular pool than to an increase in ENaC cell surface retention and likely involved 4-PBA-induced modulation of Hsc70.

**EXPERIMENTAL PROCEDURES**

**Primary Cultures of HNEC**—Nasal polyps were obtained from non-CF (n = 20) requiring surgery for their nasal polyposis as previously described (11). In addition, nasal polyps were also obtained in two CF (ΔF508/ΔF508) patients. The diagnosis of nasal polyposis was established on the basis of clinical history, endoscopic findings, and computed tomography results. This protocol was approved by the Institutional Review Board and ethics committee of our institution (CCPPRB, Hôpital Henri Mondor), and informed consent was obtained from all patients. Nasal polyp samples were immediately placed in DMEM/Ham’s F-12 supplemented with antibiotics (100 units/ml of penicillin, 100 mg/ml of streptomycin, 2.5 μg/ml of amphotericin B, and 100 mg/ml of gentamicin) and transported to the laboratory for cell isolation. Briefly, nasal polyp samples were rinsed in phosphate-buffered saline (PBS) with dithiothreitol (5 mM) and antibiotics (100 units/ml of penicillin, 100 mg/ml of streptomycin, 2.5 μg/ml of amphotericin B, and 100 mg/ml of gentamicin) and then placed overnight at 4 °C in a PBS antibiotic solution containing 0.1% Pronase. The samples were incubated in DMEM/Ham’s F-12 with 5% fetal calf serum before centrifugation (1,500 rpm, 7 min). The cell pellets were then suspended in 0.25% EDTA solution for 3 min and incubated in DMEM/Ham’s F-12 antibiotics with 10% fetal calf serum. Finally, HNEC were plated on permeable polycarbonate supports Transwell® or Snapwell® (Costar, Cambridge, MA) (1 x 10^6 cells/cm²) for short circuit current measurements. All inserts had a diameter of 12-mm and were coated with type IV collagen. HNEC were incubated at 37 °C in 5% CO₂. For the first 24 h, HNEC were incubated with 1 ml of DMEM/Ham’s F-12 antibiotics with 2% Ultroser G outside the insert and DMEM/Ham’s F-12 antibiotics with 10% fetal calf serum inside the insert. After 24 h, the medium was removed inside the inserts to place the cells at an air–liquid interface, and the medium outside the inserts was then changed daily. Transepithelial resistance and transepithelial potential difference were measured every 3 days using a microvoltmeter (World Precision Instruments, Astonbury, UK).

**Treatment of HNEC Cultures with 4-PBA**—The effects of 4-PBA in HNEC cultures on Transwell or Snapwell inserts were evaluated between days 10 and 14. The cells were treated with 5 mM of 4-PBA added to the basolateral side or with vehicle (control cells) for increasing periods of time (3, 6, or 24 h).

**Electrophysiological Studies**—Measurements of short circuit current (I sc), transepithelial potential difference, and transepithelial resistance (Rte) were performed in cells treated with 5 mM 4-PBA or the vehicle for 3, 6, or 24 h as previously described (12). Snapwell inserts were mounted in vertical diffusion chambers and were bathed with Ringer solution (pH 7.4) continuously bubbled with 5% CO₂, 95% air at 37 °C. The apical and basolateral chambers were filled with 137 mM NaCl, 5.6 mM KCl, 1.9 mM CaCl₂, 1.2 mM MgCl₂, 5.9 mM CH₃COONa, 1.3 mM NaH₂PO₄, 10 mM HEPES, and 10 mM glucose. Potential difference was short circuit to 0 mV with a voltage clamp (World Precision Instruments, Astonbury, UK) connected to the apical and basolateral chambers via Ag–AgCl electrodes and agar bridges to measure I sc. Rte was calculated by Ohm’s law. I sc was allowed to stabilize, before adding the drugs. Amiloride (10⁻⁴ M) was applied to the apical solution to calculate the amiloride-sensitive part of I sc (I sc amiloride, which is the difference between I sc measured in the absence and presence of amiloride. Amiloride-treated HNEC were then stimulated with forskolin (10⁻⁵ M, basolateral side) and IBMX (10⁻⁴ M, basolateral side) to induce cAMP–dependent Cl⁻ secretion (I sc IBMX × forsk). I sc IBMX × forsk was the difference between the initial value of I sc and the peak value obtained in response to drug addition.

Experiments were also undertaken to measure sodium influx through apical amiloride-sensitive channels in basolaterally permeabilized cells, as previously described (13). HNEC were bathed with an apical compartment solution containing 135 mM NaCl, 2.4 mM K₂HPO₄, 0.6 mM KH₂PO₄, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, pH 7.4, and a basolateral compartment solution containing 25 mM NaCl, 2.4 mM K₂HPO₄, 0.6 mM KH₂PO₄, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 5 mM glucose, pH 7.4, and 110 mM methyl-D-glucamine (an impermeant cation) for 15 min, before the basolateral membrane was permeabilized by the basolateral addition of amphotericin B (10 μM), a monovalent ionophore. This induced a rapid increase in I sc. Once the I sc reached a new steady state, amiloride (10⁻⁴ M) was added to the apical bathing solution, and the difference current, representing the amiloride-sensitive component of the sodium current across the apical membrane (I sc Δamiloride), was calculated.

**Western Blot Experiments**—Cells in Transwell filters were treated with or without 4-PBA (5 mM for 6 h), washed twice, scraped off the filters in ice-cold PBS, and centrifuged at 1500 rpm for 10 min at 4 °C. The pellet was resuspended in 500 μl of ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.1% SDS, and protease inhibitors and kept on ice for 1 h. The cell lysates were then centrifuged (12,000 rpm, 15 min) at 4 °C. Samples of the supernatants (10–15 μg of protein in 1 volume sample buffer containing 10% glycerol, 12.5% 0.05 M Tris-HCl, pH 6.8, 0.1% SDS, 5% β-mercaptoethanol, and 0.01% (w/v) bromphenol blue) were resolved through 10% acrylamide gels, electropho
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TABLE 1

Effect of 4-PBA on bioelectric measurements in HNEC cultured at air-liquid interface

| Condition     | $R_{p}$ ($\Omega$cm$^2$) | Potential difference (mV) | Basal $I_{sc}$ (µA/cm$^2$) | $I_{sc}$ amiloride (µA/cm$^2$) | $I_{sc}$ IBMX + Forsk (µA/cm$^2$) |
|---------------|-------------------------|---------------------------|-----------------------------|-------------------------------|----------------------------------|
| Controls      | 923 ± 99                | 36.9 ± 4.2                | 47.7 ± 6.1                  | 32.5 ± 5.4                    | 8.5 ± 1.5                        |
| 4-PBA (3 h)   | 1128 ± 154              | 44.6 ± 3.5                | 44.6 ± 5.9                  | 37.7 ± 5.7                    | 10.0 ± 0.9                       |
| 4-PBA (6 h)   | 950 ± 112               | 46.1 ± 4.2a               | 68.3 ± 8.8a                 | 62.6 ± 7.2a                   | 15.1 ± 1.5a                      |
| 4-PBA (24 h)  | 1127 ± 136              | 77.4 ± 3.4               | 100.4 ± 5.4a                | 97.1 ± 6.0a                   | 18.2 ± 1.6a                      |

* Significantly different from control ($p < 0.05$).
* Significantly different from control ($p < 0.01$).

HNEC grown for 10–14 days on Snapwell filters were exposed to 4-PBA (5 mM) or vehicle (control) for 3, 6, or 24 h before bioelectric measurements. Transepithelial resistance ($R_{p}$), transepithelial potential difference, and short circuit current ($I_{sc}$) were measured using a voltage-clamp system as described under “Experimental Procedures.” The data are the means ± S.E. from six to eight filters for each condition, obtained from at least five separate primary cultures.

**FIGURE 1.** Effect of 4-PBA on amiloride-sensitive sodium transport in HNEC from non-CF patients. HNEC grown on Snapwell filters were exposed basolaterally to 4-PBA or vehicle (control) for 6 h and immediately mounted into a voltage-clamp system in the presence of a Na$^+$ concentration gradient (mucosal to serosal, 135:25 mM). As shown by the representative trace in A, addition of 10 µM amphotericin B into the basolateral bath increased the $I_{sc}$ to a peak value ($I_{sc}$ amiloride, max) before amiloride (10 µM) was added into the apical bath. $I_{sc}$ amiloride represents the amiloride-sensitive component. Quantification of data is given in B. The values are the means ± S.E. of six filters for each condition. **, significantly different from control ($p < 0.01$).
Co-immunoprecipitation Experiments—HNEC were washed twice with 1 ml of cold PBS and lysed in ice-cold immunoprecipitation buffer containing 50 mM Tris-HCl, pH 7.5, 15 mM EGTA, 100 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor mix. The lysates (100 µg/ml) were centrifuged at 14,000 g for 20 min, and the pellets were discarded. The supernatants were incubated with either 5 µg of anti-α-ENaC antibody (Affinity BioReagents) or 5 µg of anti-Hsc70 antibody (Stressgen) at 4°C overnight (final volume, 500 µl). Subsequently, 50 µl of protein A-Sepharose beads was added and incubated for 1 h at 4°C. The beads were washed three times with the co-immunoprecipitation buffer. Immunoprecipitated proteins were eluted from the protein A-Sepharose beads by boiling the samples at 95°C for 5 min. The Sepharose beads were then pelleted by centrifugation.
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Reagents—Ham’s F-12 nutrient medium with DMEM, penicillin, streptomycin, amphotericin B, fetal calf serum, trypsin, EDTA, and Ultraser G were purchased from Invitrogen (Cergy-Pontoise, France). Dithiothreitol, Pronase, gentamicin, collagen IV, amiloride, forskolin, and IBMX were obtained from Sigma (Saint Quentin en Yvelines, France). 4-PBA was obtained from Calbiochem (Strasbourg, France).

RESULTS

Effect of 4-PBA on Amiloride-sensitive and cAMP-dependent Currents in HNEC—We evaluated the effects of 4-PBA on ion transports in HNEC grown on Snapwell filters by electrophysiological studies. Short circuit current measurements were performed after basolateral addition of 4-PBA for 3, 6, or 24 h and compared with controls. The mean electrophysiological values obtained from HNEC monolayers exposed or not to 4-PBA are given in Table 1. 4-PBA significantly increased \( I_{sc} \), \( I_{sc \text{amil}} \) and \( I_{sc \text{amil+fortsk}} \) at 6 and 24 h of incubation whereas 3 h of incubation had no effect. PBA affected neither amiloride-insensitive component of \( I_{sc} \) nor \( R_{sc} \) when compared with controls. To determine whether 4-PBA-induced increase in sodium transport was related to enhanced apical sodium channel activity, \( I_{sc} \) was measured after permeabilization of the basolateral membrane with amphotericin B (Fig. 1A). As shown in Fig. 1A, addition of amphotericin B to the basolateral bath rapidly increased \( I_{sc} \) to a peak value (\( I_{sc \text{ampho, max}} \)). The amiloride-sensitive part of this current (\( I_{sc \text{amil, max}} \)), reflecting sodium influx through the apical amiloride-sensitive channels, was significantly increased in HNEC exposed to 4-PBA for 6 h, as compared with controls (85.1 ± 15.2 \( \mu \)A/cm² versus 42.1 ± 12.4 \( \mu \)A/cm²; \( p < 0.01 \) (Fig. 1B).

Effect of 4-PBA on ENaC Subunit Protein Levels in HNEC—To evaluate whether the 4-PBA-induced increase in ENaC function was associated with change in \( \alpha-, \beta-, \) and \( \gamma-\)ENaC protein expression, ENaC subunit protein levels were determined by Western blot experiments in whole HNEC extracts. The anti-\( \alpha-\)ENaC antibody revealed two bands, a main band at 85 kDa and a smaller band that migrated at 65 kDa (Fig. 2A). In contrast to a recent study, using a N terminus \( \alpha-\)ENaC anti-
body, no band was detected at 30 kDa (20). However, our result is in line with a study in primary human airway epithelial cells that failed to detect this band using another N terminus antibody (21). The β-ENaC protein was detected as a single main band that migrated at 95 kDa. The γ-ENaC was detected as two bands that migrated to 85 and 75 kDa (Fig. 2A). Treatment with 4-PBA (5 mM for 6 h) did not change the protein levels of α-, β-, and γ-ENaC (normalized to the corresponding GAPDH signal) (Fig. 2B).

**Effect of 4-PBA on the Abundance of ENaC Subunits Expressed at the Cell Surface in HNEC—**Biotinylation of HNEC apical membranes was used to estimate whether 4-PBA treatment could modify cell surface expression of the three ENaC subunits. The biotinylated α-ENaC subunit protein was detected as a single band of 65 kDa, as previously reported in A6 cells derived from Xenopus laevis kidney (22) and in cultured rat alveolar epithelial type II (13) (Fig. 3A). Crude estimation revealed that the relative amount of α-ENaC protein expressed at the apical membrane of HNEC represented ~13% of total α-ENaC pool. Biotinylated β- and γ-ENaC proteins were detected as one and two bands, respectively, with molecular masses similar to the signals obtained in corresponding intracellular extracts (Fig. 3A). The relative amount of biotinylated β-ENaC and γ-ENaC expressed at the cell membrane represented ~12 and ~10% of total β-ENaC and γ-ENaC cellular pools, respectively. Exposure to 4-PBA significantly increased the amount of biotinylated α-, β-, and γ-ENaC subunits, as compared with control condition (1.85-, 2.2-, and 1.97-fold increase, respectively) (Fig. 3B). The increase in α-, β-, and γ-ENaC cell surface expression was not associated with significant change in ENaC protein levels in intracellular cell extracts. The relative abundance of ENaC proteins in intracellular extracts from control and PBA-treated cells was: 1.00 ± 0.31 versus 1.03 ± 0.33 for α-ENaC (not significant), 1.00 ± 0.10 versus 1.03 ± 0.15 for β-ENaC (not significant), and 1.00 ± 0.13 versus 0.98 ± 0.20 for γ-ENaC (not significant) (n = 6–7 independent experiments).

**Effect of Brefeldin A on 4-PBA-induced Increase in ENaC Subunit Cell Surface Expression and Sodium Transport in HNEC—**To determine whether the effect of 4-PBA on α-, β-, and γ-ENaC expression at the cell surface was related to their translocation from cytoplasmic storage to the plasma membrane, we tested the effect of brefeldin A, a fungal metabolite that inhibits intracellular trafficking of membrane proteins from the trans-Golgi network to the cell surface (17, 18) and that is used to inhibit the trafficking of ENaC subunits to the apical membrane in epithelial cells (23, 24). In control condition, brefeldin A did not change biotinylated α- and γ-ENaC proteins, whereas it tended to decrease β-ENaC protein (not significant) (Fig. 4A). Also, brefeldin A did not modify amiloride-sensitive lumen (Fig. 4B). In 4-PBA-treated cells, brefeldin A blocked 4-PBA-induced increase in α-, β-, and γ-ENaC protein levels at the cell surface (Fig. 4A) and totally prevented 4-PBA-induced increase in lumen (Fig. 4B).

**Effect on 4-PBA on α-, β-, and γ-ENaC Subunit Internalization in HNEC—**A biotinylation-debiotinylation procedure was next applied to determine whether 4-PBA-induced increase in ENaC subunit apical cell surface expression was related to a decrease in ENaC subunits retrieval from membrane to cytoplasm. Preliminary experiments were done to evaluate the endocytosis kinetics of the three ENaC subunits. Based on previous studies reporting that ENaC subunits have a short half-life in the plasma membrane (22, 25–27), we analyzed endocytosis of ENaC subunits at 30, 60, and 120 min of incubation. These experiments revealed slightly different patterns of protein internalization for α-, β-, and γ-ENaC and showed that 30 min of incubation allowed to quantify the three subunits in the same experiment (data not shown). Therefore, HNEC were biotinylated at 4 °C and replaced at 37 °C for 30 min with the MES-Na-DTT buffer before Western blotting. Quantification of internalized biotinylated α-, β-, and γ-ENaC signals was obtained using Scion Image software, and the data were normalized for the GAPDH signal in corresponding intracellular extracts. The values are the means ± S.E. of four separate experiments.
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**Effect of 4-PBA on Hsc70 and Hsp70 Protein Levels in HNEC**—The stress-inducible 70-kDa heat shock protein, Hsp70, and the constitutive 70-kDa heat shock protein, Hsc70, have been previously reported to modulate maturation, membrane trafficking, and degradation of intracellular proteins (28, 29). To evaluate whether 4-PBA could modify Hsp70 and Hsc70 protein expression, we performed Western blot in control and 4-PBA-treated HNEC. As shown in Fig. 6B, 4-PBA significantly decreased Hsc70 protein levels at 24 h, whereas it induced an insignificant decrease at 6 h. By contrast, 4-PBA did not change Hsp70 protein expression (Fig. 6A).

**Association of ENaC and Hsc70 Proteins in HNEC**—A recent study indicates that Hsc70 modulates ENaC trafficking in *Xenopus* oocytes (7). To test whether ENaC and Hsc70 proteins are associated in HNEC, co-immunoprecipitation experiments were performed. Lysates from HNEC were immunoprecipitated with antibodies directed against either α-ENaC or Hsc70 or with protein A-Sepharose beads alone. Immunoblotting with the polyclonal anti-α-ENaC antibody of lysates immunoprecipitated with anti-Hsc70 detected a strong band at 85 kDa and a faint band at 65 kDa (Fig. 7A, lane 2). These two bands were not observed when the anti-α-ENaC antibody was preincubated with excess immunizing peptide (Fig. 7A, lane 3), indicating the specificity of α-ENaC signal. Immunoblotting with the monoclonal anti-Hsc70 antibody of lysates immunoprecipitated with anti-α-ENaC resulted in a strong signal at 70 kDa (Fig. 7B, lane 2). No signal was detected in the control beads alone. These results suggest that Hsc70 was associated with ENaC in native HNEC.

**Effect of 4-PBA on Ion Transports and Cell Surface ENaC Subunit Expression in HNEC from Two CF Patients**—The effect of 4-PBA was also investigated in HNEC from two ΔF508/ΔF508 CF patients. Because few CF patients required surgery for their nasal polyposis, only two cultures of CF HNEC have been obtained. This material was used to perform electrophysiological studies (four filters for each condition) and two biotinylation experiments. Electrophysiological studies showed that CF-HNEC compared with non-CF in basal conditions have a higher amiloride-sensitive I_{sc} (42.2 ± 3.1 μA/cm^2 versus 32.5 ± 5.4 μA/cm^2, n = 4, p < 0.05) and a lower forskolin-stimulated chloride secretion (2.5 ± 2.6 versus 8.2 ± 1.4 μA/cm^2, n = 4, p < 0.01). In CF-HNEC, 4-PBA increased basal I_{sc} (87.7 ± 2.1 μA/cm^2 versus 67.0 ± 4.5 μA/cm^2 in 4-PBA and controls, n = 4, p < 0.05), I_{sc}ampl (74.2 ± 2.6 μA/cm^2 versus 42.2 ± 3.1 μA/cm^2 in 4-PBA and controls, n = 4, p < 0.05), and I_{sc}IBMX + forsk (11.0 ± 0.7 μA/cm^2 versus 2.5 ± 2.6 μA/cm^2 in 4-PBA and controls, n = 4, p < 0.01), whereas R_e was not modified (953 ± 75 Ω·cm^2 and 1169 ± 149 Ω·cm^2 in 4-PBA and controls) (Fig. 8A). Fig. 8B showed biotinylation of HNEC obtained in one CF patient. 4-PBA increased α-, β-, and γ-ENaC expression at the apical cell surface. This experiment was reproduced in HNEC from the second CF patient with identical results.

**DISCUSSION**

Phenylbutyrate (4-PBA) is a chemical chaperone that has been successfully used to promote membrane insertion of ΔF508-CFTR protein, a misfolded mutant of CFTR (8). The increased recognition of a potential role of ENaC in the pathophysiology of CF airway disease (5) led us to investigate the impact of pharmacological strategies such as 4-PBA treatment on the functional expression of ENaC. We demonstrate for the first time that 4-PBA promotes ENaC cell surface expression and activity in primary nasal epithelial cells from non-CF and CF patients. This effect is mostly related to increased translocation of ENaC subunits from the cytoplasmic pool to plasma membrane and potentially involves a regulation by 4-PBA of the constitutive form of the 70-kDa heat shock protein.

4-PBA is a transcriptional regulator with activation of numerous gene products (30) as well as a stabilizer of protein conformation that increases the rate of protein folding, accelerates protein assembly, and therefore allows the proteins to reach the membrane (31). 4-PBA has been reported to rescue a number of mutant proteins such as ΔF508-CFTR (8) but also to stabilize nonmutant proteins such as Na,K-ATPase or...
WT-CFTR (32). Here, we provide biochemical and electrophysiological evidence that 4-PBA regulates nonmutant ENaC protein cell membrane expression as well as channel activity in HNEC. Exposure of these cells to 4-PBA induced a time-dependent increase in apical amiloride-sensitive sodium current with a parallel increase in \( \alpha \), \( \beta \), and \( \gamma \)-ENaC subunit expression in the apical membrane. This result is in line with a recent study on the intestinal cell line T84, in which 4-PBA induced an amiloride-sensitive sodium transport that was otherwise not detected under basal condition (33). Indeed, previous studies in renal and airway serous cell lines have demonstrated that 4-PBA induced the increase of nonmutant Na,K-ATPase or WT-CFTR proteins in whole HNEC extracts were detected by immunoprecipitation (IP) followed by immunoblotting (IB) with the appropriate antibodies as described under “Experimental Procedures.” A, HNEC lysates were incubated with protein A-Sepharose beads alone (lane 1, control beads) or immunoprecipitated with anti-Hsc70 antibody (lanes 2–4). Immunoblotting of the gel with anti-\( \alpha \)-ENaC antibody revealed no signal in control beads (lane 1) but showed a strong band migrating at 85 kDa and a faint band at 65 kDa in immunoprecipitated lysates (lane 2). The specificity of \( \alpha \)-ENaC signal was established by the absence of signal when immunoblotting was performed in the presence of the immunizing peptide (lane 3). Immunoprecipitating with anti-Hsc70 showed a band at 70 kDa (lane 4). B, HNEC lysates were incubated with protein A-Sepharose beads alone (lane 1, control beads) or immunoprecipitated with anti-\( \alpha \)-ENaC antibody (lanes 2 and 3). Immunoprecipitation with anti-Hsc70 showed no signal in control beads (lane 1) and a band at 70 kDa (lane 4) in immunoprecipitated cell lysates (lane 2). Immunoprecipitation with anti-\( \alpha \)-ENaC showed a band at 85 kDa and a band at 65 kDa (lane 3). These experiments were repeated three times with similar results.

The increase in ENaC cell surface expression suggests a modulation by 4-PBA of ENaC protein trafficking. ENaC trafficking is complex and involves several steps; subunits are synthesized and probably assembled in a heteromultimeric complex in the endoplasmic reticulum and then processed to the Golgi (6). Only a small fraction of the subunits is expressed at the cell surface because the majority of them is rapidly degraded by the ubiquitin-proteasome pathway. ENaC subunits have a relatively short half-life in the apical membrane (25, 27). They are retrieved from the membrane by several ways including Nedd-4-mediated ubiquitination (35) and clathrin-mediated endocytosis (36). Until now, the regulation of ENaC plasma membrane density in human nasal epithelial cells either under physiological conditions or after pharmacological treatment is unknown. This study indicates that in primary human nasal epithelial cells, apical \( \alpha \), \( \beta \), and \( \gamma \)-ENaC subunits represented \( \sim 10–13\% \)
of the total $\alpha$, $\beta$, and $\gamma$-ENaC intracellular pool. 4-PBA has been previously reported to stabilize and promote correct oligomerization of proteins such as WT-CFTR, therefore preventing their rapid degradation and favoring membrane insertion. In the present study, 4-PBA-induced increase in $\alpha$, $\beta$, and $\gamma$-ENaC density and in amiloride-sensitive short circuit current was completely prevented by brefeldin A, a fungal metabolite that inhibits protein delivery to the apical membrane (17, 18). In addition, biotinylation-debiotinylation experiments indicate that $\alpha$, $\beta$, and $\gamma$-ENaC internalization was not affected by PBA treatment. Taken together, these results suggest that 4-PBA-induced increase in apical sodium channel density was due to increased incorporation of newly synthesized ENaC to the membrane but not to enhanced ENaC cell surface retention.

Previous studies have suggested that the enhancing effect of 4-PBA on CFTR trafficking is elicited by modulating the level of 70-kDa heat shock proteins (Hsc70 and Hsp70). The 70-kDa molecular chaperone has been reported to be involved in the folding and trafficking of newly synthesized proteins in the cells and to have a role in lysosomal degradation of intracellular proteins (37, 38). Recently, 70-kDa heat shock proteins have been shown to be required for the ubiquitin-dependent degradation of a number of cellular proteins such as WT-CFTR or $\Delta F508$-CFTR (10, 28, 29). These 70 heat shock proteins are composed of the constitutively expressed Hsc70 and of the stress- and heat shock-induced Hsp70. A recent work indicates that Hsc70 and Hsp70 have antagonist effects on the intracellular trafficking of ENaC (7). Herein, we demonstrate that in HNEC (i) 4-PBA decreases Hsc70 protein level but does not affect Hsp70 and (ii) Hsc70 and ENaC are associated under control condition. These data suggest that 4-PBA-mediated reduction in Hsc70 may promote the trafficking of ENaC to the cell surface. However, the direct link between decreased Hsc70 and increased ENaC cell surface expression was not demonstrated in the present study inasmuch as this would require either overexpression or silencing Hsc70, a procedure that remains difficult in native human epithelial cells. The recent study in Xenopus oocytes, showing that overexpression of human Hsc70 directly influenced ENaC cell surface density (7) supports the hypothesis of a role of Hsc70 in PBA-induced change in ENaC subunit trafficking.

Up to now, the effects of 4-PBA on ion transporters in airway epithelial cells have focused mainly on CF disease through the mutated CFTR protein (8, 39). In this mutation, the aberrant CFTR is not transported to the membrane but degraded in the ubiquitin-proteasome pathway. In CF airway epithelial cells, 4-PBA improves intracellular trafficking of $\Delta F508$-CFTR mainly by decreasing its association to Hsc70, thus leading to increased expression of CFTR at the apical membrane (10–40). Indeed, CFTR and ENaC are co-localized at the apical surface of the respiratory epithelium where CFTR can regulate ENaC activity (41, 42). In CF disease, ENaC activity appears to be increased compared with non-CF, which leads to enhanced absorption of water and electrolytes from airway surface liquid and decreased ciliary propulsion of the mucus (4). In the present study, the effects of 4-PBA were evaluated in HNEC obtained from two CF patients. In the two samples of CF HNEC, 4-PBA induced (i) a restoration of forskolin-dependent chloride secretion as previously reported in the cell line IB3-1 (8) and (ii) increased by 2-fold the amiloride-sensitive sodium transport. In addition, biotinylation experiments suggested that 4-PBA increased cell surface expression of $\alpha$, $\beta$, and $\gamma$-ENaC subunits in the apical membrane. These data indicate that in nasal epithelial cells, 4-PBA acts on the ENaC as well as CFTR protein cellular trafficking.

The present study demonstrates that 4-PBA increases the functional expression of ENaC in the apical membrane of non-CF and CF nasal epithelial cells by enhancing exocytosis of ENaC subunits likely via regulation of heat shock proteins. These results suggest that in non-CF patients, 4-PBA treatment may be useful to treat airway diseases in which ENaC trafficking may be disrupted such as during hypoxia or endotoxemia. In contrast, in CF disease, although 4-PBA may be useful to restore functional mutant CFTR at the apical membrane, it is important to keep in mind that this effect may be counteracted by an increase in sodium hyperabsorption.

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