Expression of CFTR and Cl\(^{-}\) conductances in cells of pulmonary neuroepithelial bodies

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Yeger, Herman, Jie Pan, Xiao Wen Fu, Christine Bear, and Ernest Cutz. Expression of CFTR and Cl\(^{-}\) conductances in cells of pulmonary neuroepithelial bodies. Am J Physiol Lung Cell Mol Physiol 281: L713–L721, 2001.—The pulmonary neuroendocrine cell system comprises solitary neuroendocrine cells and clusters of innervated cells or neuroepithelial bodies (NEBs). NEBs figure prominently during the perinatal period when they are postulated to be involved in physiological adaptation to air breathing. Previous studies have documented hyperplasia of NEBs in cystic fibrosis (CF) lungs and increased neuropeptide (bombesin) content produced by these cells, possibly secondary to chronic hypoxia related to CF lung disease. However, little is known about the role of NEBs in the pathogenesis of CF lung disease. In the present study, using a panel of cystic fibrosis transmembrane conductance regulator (CFTR)-specific antibodies and confocal microscopy in combination with RT-PCR, we demonstrate expression of CFTR message and protein in NEB cells of rabbit neonatal lungs. NEB cells expressed CFTR along with neuroendocrine markers. Confocal microscopy established apical membrane localization of the CFTR protein in NEB cells. Cl\(^{-}\) conductances corresponding to functional CFTR were demonstrated in NEB cells in a fresh lung slice preparation. Our findings suggest that NEBs, and related neuroendocrine mechanisms, likely play a role in the pathogenesis of CF lung disease, including the early stages before establishment of chronic infection and chronic lung disease.

cystic fibrosis transmembrane conductance regulator; expression in neuroepithelial bodies; chloride

THE PULMONARY NEUROENDOCRINE cell (PNEC) system, dispersed throughout the mucosa of the airways, is composed of single cells and innervated clusters, termed neuroepithelial bodies (NEBs; see Refs. 12 and 60). In animals and humans, NEBs express a variety of neuropeptides and a bioactive amine, serotonin (5-HT; see Refs. 10, 11, 67, 72). The expression of bombesin (BN) or gastrin-releasing peptide (GRP) in mammals is of relevance to lung development since GRP receptors have been found in the surrounding mesenchyme and submucosal glands that produce mucus secretions (19, 64). BN/GRP has mitogenic effects (34, 69), and its levels were found to be increased under a variety of pathological conditions (1). The mechanism by which expression is regulated in NEBs is not well known, but BN/GRP- and amine (5-HT; residing in dense core vesicles) are released under conditions of hypoxia (13). Evidence for an O\(_2\) sensor in NEBs has come from studies in fetal/neonatal rabbits where it has been shown that NEBs possess a membrane-localized O\(_2\) sensor complex consisting of a NADPH oxidase coupled to an H\(_2\)O\(_2\)-sensitive K\(^{+}\) channel (65). This membrane-bound sensor channel complex initiates hypoxia-induced signal transduction via activation of Ca\(^{2+}\) channels and exocytosis of amine/peptide neurotransmitters (65, 73). In the case of innervated NEBs, the hypoxia signal is transmitted to the brain stem via vagal afferents to affect control of breathing (61). In addition, local paracrine release of mediators could affect other airway functions (47).

The status of PNECs in cystic fibrosis (CF) lung disease has been examined in a few studies. Johnson et al. (30), in a series of CF cases ranging in age from 3 days to 55 yr, found up to a sixfold increase in BN/GRP-immunoreactive cells in bronchioles compared with that in normal controls and cases with prolonged mechanical ventilation. Linear hyperplasia of BN/GRP/calcitonin-positive cells was noted in a 4 yr old with CF lung disease and focal hyperplasia in a 5 yr old with CF (70).

Although cystic fibrosis transmembrane conductance regulator (CFTR) expression has been well documented in lung epithelial cells (44), there is no information about CFTR expression in PNECs, including NEBs. We present here for the first time evidence in a rabbit neonatal model that CFTR is expressed at both mRNA and protein levels in NEB cells, together with the demonstration of Cl\(^{-}\) conductances corresponding to functional CFTR in these cells. The occurrence of CFTR in the pulmonary “neuroendocrine” cell type is not surprising given that these cells are an integral component of airway epithelium, and CFTR expression has been recently demonstrated in neural cells (68). The highly specialized nature of NEB cells, including...
secretion of a variety of bioactive molecules, may impact on several aspects of the pathogenesis of CF lung disease, particularly during the early preinfectious stage.

METHODS

Lung tissue removal and preparation. New Zealand rabbits and newborns on day 2 were killed by lethal injection in accordance with Canadian Council on Animal Care guidelines, and the lungs were dissected. For whole mount staining, excised lungs were immersed in CO₂-independent medium (GIBCO BRL, Burlington, Ontario, Canada), and the lung parenchyma was removed under a dissecting microscope to leave behind intact bronchial trees. To study NEBs in situ, fresh and formaldehyde-fixed lung slices (200–400 μm) were obtained with a Leica vibratome (model 1000s).

Cell culture. T84 colon carcinoma cells, which served as a positive control for CFTR, were grown in RPMI medium supplemented with 10% FBS.

Immunocytochemistry and confocal microscopy. Immunocytochemistry was performed on vibratome sections and dissected bronchial trees using modifications of procedures previously published (65). The CFTR-specific antibodies used in this study are described in Table 1 and include the well-characterized CFTR antibody (M24-1; Genzyme), the recently introduced TAM18 monoclonal antibody against the COOH-terminal amino acid residues 1468–1480 (Neomarkers; see Ref. 9), and those provided by Transgene (MATG1061; see Ref. 45). The specificity of these latter antibodies has been described previously (41). Specificity for rabbit CFTR was verified in whole rabbit Caco-2 cell line extracts using a method published previously (41) and corresponded to the exon 13-exon 14 junction, nuclear transcripts (nt) 2481–2512 (5’-TCACCG-AAAGACAAACGACATCCACACGAAAAG-3’), and nt 2747–2777 (5’-CACAGGCAAAAGAGGAGCAACACATC-3’) of human CFTR cDNA. For normalization of total RNA, a primer pair representing human β-actin (Clontech, Palo Alto, CA) was included in the same reactions. To identify the neuroendocrine phenotype of NEB cells by RT-PCR, we amplified a fragment of the cDNA of rabbit tyrosine hydroxylase (57), the rate-limiting enzyme for 5-HT synthesis. The primer sequences were 5’-ATGATTGAAGACAATAA-3’ and 5’-TTAGATACTCGGCTTCC-3’. Commercial α-tubulin primers (housekeeping gene; Clontech) were used as loading controls. RTs were performed using the QIAGEN Omniscript RT kit as per the manufacturer’s instructions.

Electrophysiology. For electrophysiological studies, neonatal New Zealand rabbits of both sexes were used between 1 and 5 days of age. The lungs were perfused with Krebs solution and then inflated with 2% agrose (FMC Bioproducts, Rockland, ME). Transverse lung slices (150–300 μm) were cut with a Vibratome (Ted Pella, Redding, CA). Sectioning was performed with tissue immersed in ice-cold Krebs solution that had the following composition in mM: 140 NaCl, 3 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES, and 5 glucose at pH 7.3 adjusted with HCl. To identify NEB cells in fresh lung tissue, the slices were incubated with vital dye neutral red (0.02 mg/ml) for 15 min at 37°C as previously described (20, 74). For electrophysiological recordings, the lung slices were transferred to a recording chamber mounted on the stage of a Nikon microscope (Optiphot-2UD; Nikon, Tokyo, Japan). For the demonstration of voltage-activated, hypoxia-sensi-

Table 1. Characteristics of antibodies and immunochemical methods used in this study

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<tr>
<th>Anti-CFTR Antibodies</th>
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NCAM, neural cell adhesion molecule; 5-HT, serotonin; HRP, horseradish peroxidase; CARD, catalyzed reporter deposition; AB-FITC, indirect immunofluorescence using avidin-biotin complex; IF, indirect immunofluorescence.
tive K⁺ current, a feature characteristic of NEB cells, the perfusing Krebs solution for the K⁺ current recording had the following composition (in mM): 130 NaCl, 3 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 NaHCO₃, 10 HEPES, and 10 glucose at pH 7.35. When K⁺ currents were recorded, the pipette solution contained (in mM) 30 KCl, 100 potassium gluconate, 1 MgCl₂, 4 MgATP, 5 EGTA, and 10 HEPES (72). Whole cell patch recordings were performed as described by Hamill et al. (23). Hypoxia in the bath solution was achieved by bubbling the reservoir that fed the perfusion chamber with 95% N₂ and connecting the reservoir to the chamber with low-gas permeability tubing. The PO₂ level in the perfusion medium was 15–20 mmHg (20).

For assessment of Cl⁻ currents, the patch pipette was filled with (in mM) 140 N-methyl-D-glucamine (NMDG)-Cl⁻, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 1 EGTA, and 4 MgATP, pH 7.35; the bath solution contained (in mM) 140 NMDG-Cl⁻, 2 CaCl₂, 2 MgCl₂, 10 HEPES, and 1 EGTA, pH 7.35. Sucrose (100 mM) was added to the bath solution to prevent activation of chloride currents by swelling (8, 71). Because these solutions do not contain K⁺ or Na⁺, the observed whole cell currents are referable primarily to Cl⁻. Whole cell Cl⁻ currents were recorded in NEBs (neutral red positive) from fresh lung slices using the NMDG-Cl⁻ bath solution. The recording chamber (2 ml) was perfused continually with NMDG-Cl⁻ bath solution at room temperature at a rate of 6–7 ml/min.

Drugs were applied to the perfusate by switching the input flow between different channels by connecting tubing to separate flasks. Forskolin and 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) were from Sigma (Oakville, Ontario, Canada). Glibenclamide, DIDS, 5-nitro-2-(3-phenylprolamino)benzoic acid (NPPB), and IBMX were purchased from Research Biochemicals International (Natick, MA). Cl⁻ currents of NEB cells were recorded in voltage-clamp experiments with patch electrodes, which were made from 1.1-mm-OD and 0.8-mm-

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Fig. 1. A: confocal microscopy on rabbit neonatal lung airway shows neuroepithelial bodies (NEBs) stained with MOC-1 monoclonal antibody (red) and airway basement membrane highlighted by labeling with anti-laminin antibody (arrows, green). B: at higher magnification, neural cell adhesion molecule (NCAM) expression on the cell membrane is seen outlining individual NEB cells, which are clustered and have their apices extended in the airway lumen. C: typical airway NEB stained with anti-serotonin (5-HT) antibody. Magnification: A, ×200; B and C, ×300.

Fig. 2. Confocal microscopy on rabbit neonatal lung slices double stained for NCAM (rat anti-NCAM) and cystic fibrosis transmembrane conductance regulator (CFTR; red to orange signals) in NEB cells. CFTR protein, immunolocalized with the M24–1 (A), TAM18 (B), and MATG1061 (C) antibodies recognizing different epitopes (see Table 1), shows similar localization patterns at apical and basolateral membranes of NEB cells (arrows). The surrounding mucociliary epithelium expressed appreciable levels of CFTR protein as detected by catalyzed reporter deposition (CARD) amplification. A–C: magnification ×400.
ID thin-walled glass tubing (Kimax-51; Kimble) drawn with a vertical pull (PP-83; Narishige, Tokyo, Japan). An Axopatch 200B (Axon Instruments, Foster City, CA) amplifier was used for recording in the whole cell voltage-clamp mode. The data were acquired with amplifier filters set at 5 kHz. The level of the fluid over the slices was kept low to minimize the time constant of the patch electrodes. Voltage commands and data acquisition were done using pClamp 6 software and a DigiData 1200 interface (Axon Instruments). The leak current was subtracted from all current records using pClamp software. All data values are given as means ± SE. Statistical analysis was performed using the paired and unpaired Student's t-test. Differences were considered to be statistically significant at P < 0.05.

RESULTS

CFTR localization in lung cells as determined by confocal microscopy. Confocal microscopy on lung slices was employed to discriminate double labeling of NEBs with combinations of antibodies. Antibodies to NCAM epitopes, both MOC-1 and the rat anti-mouse NCAM antibody, labeled the plasma membrane surrounding individual neuroendocrine cells within the NEBs (Fig. 1, A and B). We used an anti-laminin antibody to outline the bronchiolar basement membrane and showed that the NEB surface is exposed to the airway lumen (Fig. 1A). The surrounding mucociliary epithe-

Fig. 3. Light-microscopic images of the localization (A), picking (B), and isolation (C) of NEBs from fresh lung slices after visualization with neutral red. DEPC, diethyl pyrocarbonate. A–C: magnification ×100.
lum did not stain with NCAM-specific antibodies. NEBs were also identified by their content of 5-HT, as demonstrated with a specific antibody to 5-HT (Fig. 1C).

With respect to CFTR localization, the MATG1061, TAM18, and the M24–1 monoclonal antibodies all labeled mucociliary epithelial cells in lung slices (Fig. 2, A–C). Initial indirect immunofluorescence labeling on
cryosections revealed strong apical membrane staining of CFTR in mucociliary epithelium and weaker signals in NEB cells (data not shown). To enhance detection of CFTR in NEB cells, we applied the method of CARD amplification, which increased detection of CFTR protein in both the cytoplasm and plasma membrane. No labeling for CFTR was seen in the submucosal connective tissue surrounding the bronchial epithelium. Compared with the surrounding epithelium, the level of apparent CFTR expression in neonatal NEBs was lower and was not detected on all cells within the NEB clusters. Dual labeling with the NCAM neuroendocrine marker and visualization by confocal microscopy confirmed the overlapping signals, indicating coordinate expression (Fig. 2, A–C). In summary, the confocal-microscopic studies indicated that NEBs in rabbit neonatal lung express CFTR protein at apical and lateral plasma membranes with low to moderate levels.

Expression of mRNAs for tryptophan hydroxylase and CFTR in NEBs using RT-PCR. To confirm that CFTR is expressed in NEB cells, we applied RT-PCR to NEB clusters that were removed using patch-clamp pipettes (Fig. 3, A–C). T84 cells served as a positive control for CFTR message expression, and rabbit cortical brain served as a positive control for expression of tryptophan hydroxylase (TH), a marker of NEBs, since it is the rate-limiting enzyme for synthesis of 5-HT.

Figure 4A shows a 295-bp PCR product for CFTR in T84 cells but not in rabbit brain and moderate to strong PCR signals in neonatal NEBs and neonatal bronchial epithelial cells picked from adjacent regions negative for neutral red staining. The neuroendocrine nature of isolated NEBs was confirmed by RT-PCR for TH mRNA (Fig. 4B). The large 1,340-bp TH PCR product was correspondingly obtained for neonatal NEBs from lung slices in rabbit brain but not in T84 cells or in neutral red-negative neonatal bronchial epithelial cells. β-Actin and α-tubulin RT-PCRs served as controls for loading and fidelity of the reactions.

To exclude the possibility that picked NEBs entrapped pulmonary epithelial cells that could account for a CFTR signal by RT-PCR, we used a suction patch pipette to extract cytoplasmic contents from single NEB cells and applied the method of single-cell RT-PCR (54, 59, 63). Results were obtained (data not shown) that confirmed the expression of CFTR mRNA in single NEB cells.

Electrophysiology. We first established that the neutral red-positive NEBs in lung slices exhibited voltage-gated K⁺ currents, as reported previously (74). Depolarizing steps from a holding potential of −60 to +30 mV evoked outward K⁺ currents in the majority of the cells tested (95%; Fig. 5A). Again, as previously reported, the exposure to a hypoxic stimulus resulted in a rapid and reversible reduction in amplitude and time course of the outward K⁺ currents in NEB cells. Current amplitudes at +30-mV test potential were reduced by 34% after hypoxic exposure (Figs. 5B). Washout of the hypoxic solution led to recovery of the outward K⁺ current (Fig. 5C).

In a separate set of lung slices, we measured Cl⁻ currents in neutral red-positive NEBs. Whole cell Cl⁻ currents in unstimulated NEB cells were around ± 500 pA at ± 70 mV (n = 19) and remained stable. During Cl⁻ current recordings, the membrane potential was held at 0 mV, and currents were evoked by voltage steps from −80 to +80 mV. The reversal potential was −4.5 mV, near 0 mV, as expected for a Cl⁻ current. There was no time dependence of the currents during hyperpolarizing voltage pulses (Fig. 5, D and G). The mean control Cl⁻ current was 463.8 ± 50 pA (n = 5) at +70-mV potential. After perfusion with the Cl⁻ channel blocker NPPB (100 μM; an inhibitor of epithelial Cl⁻ channels), the mean current decreased to 340.2 ± 40 pA (n = 5), a reduction of 27% (Fig. 5G). After perfusion with a cocktail of cAMP solution (10 μM forskolin, 100 μM CPT-cAMP, and 1 μM IBMX), the mean Cl⁻ current was 662.4 ± 40 pA (n = 5, P < 0.05) at +70-mV potential, and compared with control Cl⁻ currents, it produced sustained increases in basal current by 46% (n = 10, P < 0.05; Fig. 5, E and G). The mean Cl⁻ current was 354.7 ± 30 pA (n = 6, P < 0.05) after perfusion with the selective inhibitor glibenclamide (50 μM), an inhibition of 29% relative to control at +70 mV (Fig. 5, F and H). Perfusion with DIDS (200 μM), an inhibitor of outward rectifying Cl⁻ channels (Fig. 5H), yielded a mean Cl⁻ current of 441.8 ± 30 pA (n = 4), a minimal difference compared with control.

DISCUSSION

Our studies demonstrate for the first time that CFTR is expressed in an airway cell type that has not been previously considered as a potential contributing component in the pathobiology of CF lung disease. Anti-CFTR antibodies have been used extensively in a variety of studies to demonstrate expression of CFTR protein in lung epithelium and other tissues (2, 4–7, 15, 16, 21, 24–27, 29, 31, 37, 39, 43, 76), including fetal rabbit (40). In agreement with the above studies, CFTR protein was also localized to the apical region of postnatal rabbit lung epithelial cells. Enhancement with the CARD system identified abundant CFTR protein in epithelial cells and was necessary to demonstrate expression in NEB cells. Overall, CFTR protein levels in NEBs appeared lower compared with that in surrounding mucociliary epithelium. In isolated NEBs and in single NEB cells, RT-PCR confirmed expression of CFTR mRNA. Patch-clamp analysis confirmed the presence of a CFTR Cl⁻ conductance in NEB cells. Thus pulmonary NEBs should now be included among a growing number of cell types that express functional CFTR, including thyroid (16), endothelium (58), pancreas (37, 76), intestine (21, 25), and brain (26, 43). Taken together, the patterns of tissue-specific CFTR expression suggest that this Cl⁻ channel function may be involved in a variety of physiological processes.

From a physiological point of view, PNECs, both single cells and NEBs, are of great interest, since a wide variety of neuropeptides and amines are ex-
pressed in these cells (11, 55, 60). Several of the peptide products, particularly BN, exhibit a range of physiolog-ical and cellular functions, including mitogenesis, bronchial smooth muscle contraction, vasoactivity (34), mucus secretion (3), branching morphogenesis (33), and as a chemoattractant (14). How the expression of the neuropeptides in PNECs and NEBs is controlled is not known, but recent evidence suggests that epithelial-mesenchymal interactions are important as well as hypoxia (11, 49). We have shown that NEBs possess an O$_2$ sensor function that consists of an O$_2$-sensitive NADPH oxidase complex coupled to a H$_2$O$_2$-sensitive K$^+$ channel (65, 73, 74). Thus it is postulated that this signaling complex depolarizes the NEB cell under conditions of hypoxia to trigger Ca$^{2+}$ influx and subsequent release of neuropeptides and 5-HT in the surrounding tissues and vasculature (12).

The potential involvement of PNECs in CF lung disease processes is still not understood. However, reports of PNEC hyperplasia in lungs of CF patients (30) and detection of significant amounts of BN-like peptide (BLP) in urine from CF patients (46) suggest an overactive neuroendocrine cell system. In contrast to the age-dependent decrease of BLP in urine of normal humans, BLP levels in urine from CF patients were found to be elevated for up to 5 yr of age (46). Thus BLP in urine from CF patients may reflect increased PNEC activity. Because BN/GRP possesses mitogenic properties on lung mesenchyme (48, 56) and regeneration of pulmonary epithelium (e.g., Clara cells) appears to focus around NEBs that are located at bifurcations (51), it is possible that, in lungs of infants with CF, NEBs via secretion of neuropeptides are essential for maintenance of the bronchopulmonary epithelium either directly or through interaction with adjacent mesenchyme. Receptors for GRP can be found in the surrounding mesenchymal cells and also in the submucosal gland ductal cells in the lung (64). A defect in CFTR function is thought to lead to thickening of the mucus produced by the submucosal glands as it tries to exit the ducts, whereas ductal plugging is seen in CF lungs (17). Thus BN/GRP acting through its receptor may be involved in this process. During this early phase in CF lung disease, inflammatory changes without infection are evident (18, 42), with cytokines such as interleukin-8 playing a role in recruitment of neutrophils (42). Immune cells have been noted to associate with NEBs (62), and other factors such as tumor necrosis factor-$\alpha$ may be involved in PNEC differentiation (22).

Pulmonary disease is the major cause of morbidity and mortality in patients with CF and is largely the result of a scenario of overwhelming infections, compromised host defense mechanisms, and ineffective antibiotic treatment (44). The pathogenesis underlying this sequence of events is thought to involve hyperactive mucous cells and submucosal glands (35, 52), impaired mucociliary clearance of a thickened mucus, and possibly a compromised pulmonary cell defense system, all resulting from loss of CFTR functions (44). This may facilitate overgrowth of nonpathogenic bac-

terial strains in healthy lungs (38). Although bacterial infection is a prominent feature of CF lung disease, during the early stages of the disease (i.e., postnatal period), infection is minimal, and other factors may play more important roles in establishing the pathogenic process (32).

Our observations that CFTR mRNA and protein are expressed in NEBs postnatally, that NEBs possess Cl$^-$ conductance channels with CFTR characteristics, and the demonstration that CFTR may modulate other channels (36, 53) all lend support to the idea that CFTR could have a physiological role in NEBs. Further studies are needed to determine if NEB function, such as the autocrine/paracrine modulation that figures prominently within the first few months of life, is compromised by defective CFTR at a time when the infectious process is minimal and mucociliary functions are maturing.

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REFERENCES


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CFTR EXPRESSION IN PULMONARY NEUROENDOCRINE CELLS


