Ion transport in epithelial spheroids derived from human airway cells

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Ion transport in epithelial spheroids derived from human airway cells. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L75–L80, 1999.—In the present study, we describe a novel three-dimensional airway epithelial explant preparation and demonstrate its use for ion transport studies by electrophysiological technique. Suspension cultures of sheets of epithelial cells released by protease treatment from cystic fibrosis (CF) and non-CF nasal polyps developed free-floating, monolayered epithelial spheres, with the apical, ciliated cell membrane facing the bath and the basolateral cell membrane pointing toward a fluid-filled lumen. Microelectrode impalement of both non-CF and CF spheroids revealed lumen-positive transepithelial electrical potential differences (PDs) that were inhibited by amiloride, indicating that the spheroids were inflated due to amiloride-sensitive Na⁺ absorption followed by water. Transformation to a Cl⁻ secretory state was achieved by addition of ATP to the bath, leading to the development of a diphenylamine-2-carboxylate-sensitive PD. A cAMP-induced increase in PD was seen in non-CF spheroids only. In response to hydrocortisone treatment, Na⁺ transport reflected by amiloride-sensitive PD increased and more so in CF than in non-CF spheres. We concluded that this preparation is a useful model for the airway surface epithelium and is suitable for studies of transport mechanisms and regulation.

Materials and Methods

Cellular material. Nasal polyps were resected from 17 normal subjects (7 women and 10 men) and 9 CF patients (4 men and 5 women), of which 8 were ΔF 508 homozygous and 1 had the ΔF 508/ΔF 2098-1G-C combination. The polyps were placed in a 10-ml test tube with ~8 ml of Dulbecco’s modified Eagle’s medium (room temperature, pH 7.30–7.40) containing 0.1% protease type XIV (Sigma, St. Louis, MO), 10⁵ U/l of penicillin, 100 mg/l of streptomycin, and 50 μg/ml of gentamicin. Sheaths of epithelial cells were dislodged from the polyps by intermittent shaking. After 1–2 h, fetal bovine serum (10% vol/vol; GIBCO, Grand Island, NY) was added to neutralize the protease. The solid parts of the polyps were isolated and discarded, and the remaining epithelial suspension was washed twice (5 min at 110 g) in Ham’s F-12 culture medium containing 1% Ultroser-G serum substitute (IBF Biotechnics, Savage, MD) and antibiotics as above. The pellet was resuspended in 5 ml of culture medium in a 50-ml tissue culture bottle, changed every 3–4 days after gentle centrifugation (20 g for 2 min). From the third day on, gentamicin was omitted from the medium. In a series of experiments, hydrocortisone (HC; 5 x 10⁻⁶ M) was added 1–2 days before electrophysiological experiments.

Scanning electron microscopy. The epithelial spheroids were transferred to 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. After fixation for 24 h at 5°C and a short rinse in 0.15 M cacodylate buffer (pH 7.4), the specimens were postfixed in 1% OsO₄ in 0.12 M cacodylate buffer (pH 7.4) for 1 h, rinsed in distilled water, and prepared for scanning electron microscopy examination by the osmium-thiocarbohydrazide method, consisting of a thorough 1-h rinse in distilled water, a 30-min treatment with a saturated and filtered solution of thiocarbohydrazide in distilled water, and a second continuous rinse in distilled water, and then the specimens were returned to 1% OsO₄ for 30 min. The latter four steps were repeated, and after a final rinse in distilled water, the specimens were gradually dehydrated to 100% ethanol within 24 h, transferred to 100% ethanol for 1 h, and critical point dried (Balzers CPD 030, Belzer Union, Liechtenstein) with...
CO₂. Specimens were mounted on stubs with adhesive carbon tabs and sputter coated with chromium (XE200 Xenosput, Edwards High Vacuum, Crawley, UK). Examination and photography were carried out in a Philips FEG 30 scanning electron microscope operated at 0.5–5 kV.

Transmission electron microscopy. After fixation and post-fixation as described in Scanning electron microscopy, the specimens were centrifuged to form a pellet and embedded in agar. The samples were dehydrated in a graded series of ethanol, transferred to propylene oxide, and embedded in Epon. Sections were cut with a Leica Ultratcut UCT microtome, collected on one-hole copper grids with Formvar-supporting membranes, stained with uranyl acetate and lead citrate, and examined and photographed in a Philips EM 208 transmission electron microscope operated at an accelerating voltage of 80 kV.

Electrophysiology. The transepithelial PD was measured in epithelial spheroids transferred to 700 µl of HEPES-buffered Ringer solution [containing (in mM) 140 Na⁺, 5 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 131.2 Cl⁻, 1.6 HPO₄²⁻, 0.4 H₂PO₄⁻, 10 glucose, and 10 HEPES, titrated to pH 7.35] at 37°C in a thermostat-controlled chamber placed on the stage of an inverted microscope (Litz). Spheroids were kept in position by applying gentle suction with a Ringer-filled holding pipette (Swemed Lab, Billdal, Sweden) with an internal tip diameter of ~25 µm. Spheroids were impaled by microelectrodes pulled from filamented borosilicate glass tubes (OD 1 mm; Clark Electromedical, Reading, UK) on a horizontal puller (P-87, Sutter Instruments, Novato, CA) and backfilled with 0.3 M KCl (tip resistance 70–100 MΩ). The holding pipette and microelectrode were operated by micromanipulators, and spheroid microelectrode impalement was provided by a piezo translator (MPM-10, World Precision Instruments, Sarasota, FL). The microelectrode was connected to a high-impedance electrometer (Duo 773, World Precision Instruments, Sarasota, FL).}

**RESULTS AND DISCUSSION**

Sheets of epithelial cells from CF and non-CF nasal polyps released by protease treatment and cultured in a defined, serum-free medium formed fluid-filled spheroids with diameters of 50–800 µm. From each resected polyp (pea size), >100 spheroids were formed, ready to be used for electrophysiological experiments within only a couple of days after resection. It should be pointed out that the spheroids represent explants rather than the result of a primary culture procedure. Accordingly, no mitotic figures were observed in the epithelial cells at any time.

The yield of cells in spheroids by the present method seems to be rather high. Thus, for example, 100 spheroids with a diameter of 300 µm represent an epithelial area of ~0.3 cm², which corresponds to a significant fraction of the surface epithelium of a nasal polyp (1–1.5 cm²). Furthermore, the number of experimental preparations resulting from a single polyp is higher (>100) with the present method than with the traditional primary culturing of cells in flat sheets. However, the number of cells (e.g., for biochemical studies) obtained in spheroids is of course smaller than after upformation of primary cultures.

The epithelial morphology of the spheroids, which was stable for several weeks of continuous culture, resembled that of the native tissue and collagen-supported cell culture (6, 10, 13). Thus all spheroids consisted of a fully differentiated monolayered epithelium, with the apical cell membrane containing stubby microvilli facing the bath and the basolateral membranes pointing toward the central, clear, fluid-filled lumen (Figs. 1 and 2). We were unable to distinguish between CF spheroid (CFS) and non-CFS (NCFS) morphology. A large part of the cells carried cilia located exclusively on the apical side (Fig. 1), and ciliary activity and associated spheroid movements were present during the entire observation period.

The average size of individual spheroids appeared to be almost constant until final collapse. No differences in size were observed between the CFSs and NCFSs. The diameter of six isolated NCFSs, measured daily from the age of 2 wk until collapse 2–4 wk later, fluctuated 23–41% around the average diameter (225 µm). Such volume fluctuations might reflect the inflation of the spheroids due to an inward fluid transport, interrupted by periods of shrinkage caused by stretch-induced opening of the tight junctions (17). The presence of an active inward solute and water transport, resulting in an increased intraluminal pressure, was supported by an observed shrinkage after deliberate puncture of the spheroids, in which the diameter was reduced to ~75% of the original value. Most likely, this reflects an active inward transport of Na⁺ via apical membrane Na⁺ channels and basolateral membrane Na⁺-K⁺ pumps (25), giving rise to NaCl accumulation in the closed inner compartment followed by an inward flux of water due to osmotic coupling.

To test this hypothesis, we measured the transepithelial PD by introducing microelectrodes into the lumen of the spheroids (Fig. 3). By this procedure, we found positive intraluminal (basolateral) PDs ranging from 2.2 to 14.5 mV, with no significant difference between CFSs and NCFSs. When 100 µM amiloride was added to the bath (apical side), the PD of CFSs was abolished (from 6.1 ± 0.4 to 0.0 ± 0.1 mV; n = 29 spheroids), whereas the PD of NCFSs was reduced by ~60% (from 6.9 ± 0.5 to 2.5 ± 0.3 mV; n = 35 spheroids). These results confirm the notion that amiloride-sensitive Na⁺ absorption is the main determinant of airway epithelial PD (25). The difference in responses to amiloride between NCFSs and CFSs is also well known (12). It probably reflects that amiloride hyperpolarizes the apical cell membrane, which in NCFSs induces a small Cl⁻ secretion, whereas this possibility is absent in CFSs because of the missing expression of the CF transmembrane conductance regulator (CFTR) in the apical cell membrane. The finding that control PD values were equal in NCFSs and CFSs contrasts with a previous study (10) where the PD of CF airway epithelia was higher than that of non-CF airway epithelia.
a few studies (24, 28, 29), however, identical non-CF and CF PD values have been reported. The explanation for these discrepancies is not clear but may involve differences in epithelial resistance in response to different experimental conditions or differences in hormonal stimulation of transport mechanisms (see below). A detailed quantitative analysis of transport properties in epithelia includes measurements of epithelial resis-

Fig. 1. A: scanning electron microscopy of an ~250-μm epithelial spheroid after 4 wk in culture medium. B and C: considerable number of epithelial cells are covered with cilia (arrows), and remainder of cell membrane is equipped with stubby microvilli (arrowhead). Magnifications and bar lengths: ×750, 100 μm (A); ×9,000, 10 μm (B); and ×22,500, 5 μm (C).

Fig. 2. Transmission electron microscopy survey of epithelial cells. A: outer (apical) cell membrane (O) faces culture medium (M) and is equipped with microvilli (solid arrowheads). Inner (basolateral) cell membrane (I) faces fluid-filled lumen (L); no basal membrane was observed. Epithelial cells are joined with zonula occludens junctions toward the culture medium (box), and lateral cell membranes are closely opposed (arrows). Each epithelial cell contains a nucleus (N), numerous mitochondria (Mi), endoplasmic reticulum studded with ribosomes (open arrowheads), Golgi complexes (G), and lipid inclusions (Li). Magnification, ×17,000; bar, 1 μm. B: high magnification (×144,500) of framed area in A displaying the terminal bar region of 2 epithelial cells consisting of a zonula occludens with 3 sealing strands (arrowheads). Bar, 0.1 μm.
tance (to calculate equivalent short-circuit current) and cellular PDs with conventional and ion-selective microelectrodes (to calculate ionic driving forces). Such measurements are under current development in the present spheroid preparation. However, because natural upper airway epithelia are low-resistance epithelia (20), it is unlikely that the presently observed drug-induced changes in PD can be explained merely by changes in paracellular resistance; furthermore, the PD changes resemble those observed in other preparations.

During formation of the spheroids in Ham's F-12 culture medium, this medium is trapped in the interior (basolateral compartment). Regarding ions of major importance (Na$^+$, Cl$^-$, and K$^+$), this medium has a composition close to that of the Ringer solution used as the external (apical) medium in the present study. Thus significant contributions of diffusion potentials to the transepithelial PD resulting from asymmetric ion concentrations are unlikely. This is further supported by the observation that the transepithelial PD is zero when active Na$^+$ absorption is blocked by amiloride and Cl$^-$ secretion is absent (CFSs).

After the addition of amiloride, the residual PD of NCFSs was sensitive to the addition of the Cl$^-$-channel blocker 2-DPC (250 µM) (5a), which reduced the residual PD by 60.0 ± 8.4%. In contrast, the addition of 2-DPC to NCFSs before amiloride had no effect on PD. This is in accordance with the hypothesis that 2-DPC-sensitive, unstimulated apical membrane Cl$^-$ outflux in non-CF airway epithelium is triggered by membrane hyperpolarization caused by amiloride (26). The pathway for this 2-DPC-sensitive Cl$^-$ outflux is obviously related to CFTR because no effect of 2-DPC on PD (before or after amiloride) was observed in the CFS. The Cl$^-$-channel blocker DIDS (400 µM) proved to be without any significant effect on PD before or after amiloride addition in both CFSs and NCFSs.

In a series of experiments, amiloride-treated spheroids were exposed to 200 µM ATP to investigate the possible presence of an ATP-sensitive Cl$^-$ channel in the apical cell membrane (4, 11). An abrupt rise in the basolateral side positive PD followed by a decrease to a still increased steady level was observed in both CFSs and NCFSs in response to ATP addition to the bath (Fig. 4). However, in contrast to previous reports (4, 11), the change in PD was not larger in CFSs than in NCFSs (Fig. 4). The sustained level was insensitive to DIDS but sensitive to 2-DPC in both NCFSs [change in

![Fig. 3. Microelectrode impalement of spheroid. Left, holding pipette with internal diameter of ~25 µm. Right, microelectrode.](image)

![Fig. 4. A: summary of transepithelial potential difference (PD) measurements in epithelial spheroids (not pretreated with hydrocortisone) from 5 cystic fibrosis (CF) and 5 non-CF patients (not pretreated with hydrocortisone). All spheroids went through the following sequence of drugs added to the bath (apical side): 100 µM amiloride, 200 µM ATP, and 250 µM diphenylamine-2-carboxylate (2-DPC). Data are means ± SE; n, no. of spheroids. Mean steady-state PD in control medium was 6.6 ± 0.6 mV for CF spheroid (CFS) group and 6.6 ± 0.7 mV for non-CFS (NCFS) group. Amiloride nullified PD of CFS group to 0.1 ± 0.1 mV and reduced PD of NCFS group to 2.5 ± 0.6 mV. Subsequent addition of ATP increased PD in both NCFSs and CFS groups, reaching maximum peak values (ATP spike) of 6.7 ± 0.8 (NCFS) and 3.9 ± 0.6 mV (CFS), which were succeeded by reduced, but still increased, sustained PDs [4.8 ± 0.7 (NCFS) and 1.4 ± 0.3 mV (CFS); ATP steady]. With addition of 2-DPC, these remaining PDs were reduced to 0.5 ± 0.5 (NCFS) and 0.6 ± 0.2 mV (CFS). Significant difference from respective NCFS group: *P < 0.02; **P < 0.002 by unpaired Student's t-test. NS, not significant. B: original trace demonstrating effects on PD of same sequence of drug applications. I, microelectrode impalement of spheroid; O, withdrawal of microelectrode from spheroid.]
PD ($\Delta PD = -4.3 \pm 0.3 \text{ mV}; n = 11$) and CFSs ($\Delta PD = -0.8 \pm 0.2 \text{ mV}; n = 14$). Pretreatment with 2-DPC, but not with DIDS, prevented a subsequent ATP response. In six experiments, a brief negative deflection in PD preceded the usual initial positive spike, indicating a short-lasting ATP-dependent activation of conductances other than that carrying Cl$^-$, such as K$^+$ (5).

During individual micropuncture experiments lasting 20–30 min, we were unable to detect fluid transport-dependent volume changes of the spheroids from the video frames, primarily because the microscope focusing was not continuously at the plane of the spheroid perimeter. Furthermore, it was difficult to detect spheroid volume changes from short-lasting diameter measurements if unstimulated fluid absorption in this preparation was as low as in primary cultured human nasal epithelium (8, 21) or in human bronchial xenografts (29), i.e., 0.1–4 µl·cm$^{-2}$·h$^{-1}$. Finally, the experimental protocol did not allow observations of unperturbed spheroids for >10 min before inhibition of absorption by amiloride and stimulation of secretion.

The dominating amiloride-sensitive Na$^+$ absorption in airway epithelium is subject to long-term regulation, probably by steroid hormones (3). To test this possibility, we measured the PD of NCFSS and CFSs after addition of HC to the incubation medium 1–2 days before electrophysiological measurements. The presence of HC resulted in significantly larger basolateral side positive PDs than in the absence of hormone (see above) and more so in CFSs [18.0 ± 0.8 mV ($n = 19$)] than in NCFSS [11.4 ± 0.8 mV ($n = 26$)] ($P < 0.01$; $n = 35$). These larger control PD values doubtlessly represent increased levels of amiloride-sensitive Na$^+$ absorption because apical amiloride (10$^{-4}$ M) decreased PD to the same level as in the absence of HC (see above). Thus, in the presence of HC, amiloride decreased the PD in NCFSS from 11.4 ± 0.8 to 2.0 ± 0.1 mV ($n = 19$). Furthermore, the data suggest that the previously reported upregulation of amiloride-sensitive Na$^+$ channels in CF airway epithelial cells lacking CFTR may be influenced by hormonal stimulation, in this case by HC (3).

cAMP has been demonstrated to activate an apical Cl$^-$ conductance in epithelial cells expressing CFTR (1). In normal airway epithelia, this may result in cAMP-stimulated Cl$^-$ secretion, whereas a similar stimulation in CF epithelia is not possible because of the lack of CFTR. In accordance with this notion, we observed a small but significant increase in the PD of HC-stimulated and amiloride-treated NCFSSs (from 2.2 ± 0.3 to 3.1 ± 0.4 mV; $P < 0.001$; $n = 26$) 2–3 min after addition of 0.25 mM dibutyl cAMP and 0.25 mM IBMX to the apical bath, whereas the PD of CFSs did not respond to this treatment (from 0.0 ± 0.1 to 0.1 ± 0.1 mV; not significant; $n = 19$). The rather small increase in PD of NCFSSs may be related to the fact that the driving force for Cl$^-$ across the apical cell membrane is small even in the presence of amiloride (26). Subsequent apical application of 200 µM ATP stimulated the basolateral side positive PD slightly more in CFSs than in NCFSSs ($P = 0.13$). Thus, in CFSs, the maximal PD increased from 0.1 ± 0.1 to 9.4 ± 1.6 mV ($\Delta PD = 9.3 ± 1.6 \text{ mV}; P < 0.001; n = 19$) and in NCFSSs from 2.9 ± 0.4 to 8.7 ± 1.1 mV ($\Delta PD = 5.8 ± 1.0 \text{ mV}; P < 0.001; n = 19$).

This first study of functional characteristics of spheroid-shaped airway epithelial explants demonstrates that it is possible, from small amounts of primary material, to rapidly make large numbers of differentiated ready-to-use preparations of non-CF and CF airway epithelium with electrophysiological properties resembling those of the native tissue. The spheroid preparation may be suitable for application of several additional types of experimental techniques. The relative inaccessibility of the enclosed basolateral compartment may be a drawback. However, this medium may be accessible for applications or sampling of, e.g., agonists, tracers, and metabolites by use of high-pressure vacuum micropipettes and iontophoresis or by centrifugation of the spheroids out of suspension. Furthermore, the structural arrangement of the epithelium without underlying distracting supports may represent a unique possibility to reach the serosal cell membrane for impalements or patch clamping after opening of the spheroid. Thus this preparation is an experimental model useful for investigations of regulatory mechanisms of airway epithelial transport functions.

We thank N. J. Brandt, J. Rostgaard, R. Sinding, and N. Rasmussen for help and encouragement during this study. This work was supported by grants from the Danish Research Council of Health Sciences, the Novo-Nordisk Foundation, the Danish Heart Association, and the Velux Foundation.

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Received 23 September 1997; accepted in final form 18 September 1998.

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