Fluid absorption related to ion transport in human airway epithelial spheroids

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Pedersen, Peter Steen, Niels-Henrik Holstein-Rathlou, Per Leganger Larsen, Klaus Qvortrup, and Ole Frederiksen. Fluid absorption related to ion transport in human airway epithelial spheroids. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L1096–L1103, 1999.—Airway epithelium explants from cystic fibrosis (CF) patients and non-CF subjects formed monolayered spheres, with the apical ciliated cell membrane facing the bath and the basolateral cell membrane pointing toward a fluid-filled lumen. With the use of two microelectrodes, transepithelial potential difference and changes in potential difference in response to passage of current pulses were recorded, and epithelial resistance and the equivalent short-circuit current were calculated. Non-CF control potential difference and short-circuit current values were significantly lower than the CF values, and amiloride inhibited both values. Fluid transport rates were calculated from repeated measurements of spheroid diameters. The results showed that 1) non-CF and CF spheroids absorbed fluid at identical rates (4.4 µl·cm⁻²·h⁻¹), 2) amiloride inhibited fluid absorption to a lower residual level in non-CF than in CF spheroids, 3) Cl⁻–channel inhibitors increased fluid absorption in amiloride-treated non-CF spheroids to a level equal to that of amiloride-treated CF spheroids, 4) hydrochlorothiazide reduced the amiloride-insensitive fluid absorption in both non-CF and CF spheroids, and 5) osmotic water permeabilities were equal in non-CF and CF spheroids (~27 × 10⁻⁷ cm·s⁻¹·atm⁻¹).

amiloride; hydrochlorothiazide; osmotic permeability; current-voltage relationship; cystic fibrosis

The depth of the periciliary fluid layer (5–10 µm) in the upper airways (bronchi, trachea, and nasal cavity) is of paramount importance for ciliary activity and, thereby, for mucociliary clearance in the airways. The mechanisms by which both the thickness and composition of this fluid layer are regulated are still largely unknown. Fluid may be added from distal airways, submucosal glands, and surface epithelium secretion. On the other hand, fluid may be removed and the composition may be changed by mucociliary clearance, evaporation, and, importantly, surface epithelium absorption (3, 4). Changes in more than one of these mechanisms seem to be involved in the pathogenesis of cystic fibrosis (CF), characterized by a defective gene product leading to missing cAMP-dependent Cl⁻–channel function and increased activity of amiloride-sensitive Na⁺ channels (4). During the last few years, two views have evolved for the regulation of volume and composition of the airway surface fluid layer (ASL), leading to different predictions regarding the physiology of CF and non-CF airway epithelia. One hypothesis predicts that the ASL in CF epithelium is more hypertonic than that in non-CF epithelium, which may inhibit the function of a bacterial defense mechanism in CF (11, 29). The second hypothesis predicts that the volume of ASL in CF epithelium is decreased compared with the non-CF epithelium, resulting in a decrease in mucociliary clearance (16). These hypotheses are derived from the diverging results obtained by different techniques for the measurement of volume and composition of the pericellular fluid and transepithelial solute transport. The development of an additional experimental model for each of the components, especially fluid transport might contribute to the knowledge of these controversial topics.

We (18) recently described the structural and basic ion transport characteristics of spheroid-shaped explants of human airway epithelium from CF patients and normal (non-CF) subjects. Suspension-cultured sheets of protease-released epithelium derived from resected nasal polypos formed free-floating, matrix-independent, monolayered epithelial spheres, with the apical ciliated cell membrane facing the bath and the basolateral cell membrane pointing toward a fluid-filled lumen. Measurements of the transepithelial potential difference (PD) with microelectrodes demonstrated that the spheroid explant preparation discloses ion transport characteristics similar to those previously demonstrated in CF and non-CF airway epithelia (3, 4).

In the present study, the capacity of the dominating Na⁺ absorption was evaluated in non-CF (NCFSs) and CF (CFSs) spheroids from measurement of the equivalent short-circuit current (Iₑ) before and after application of amiloride. Furthermore, the ability of these airway epithelial preparations to absorb fluid was determined from repeated measurement of spheroid diameters, and the relationship between ion and water transport was studied with inhibitors of Na⁺ absorption and Cl⁻ secretion. Finally, the ability of the airway explant preparations to maintain osmotic gradients was evaluated from the measurement of hydraulic permeability.
MATERIALS AND METHODS

Cellular material. Nasal polyps were resected from 15 non-CF subjects (8 women and 7 men) and 7 CF patients (5 men and 2 women; all ΔF508 homozygous). The procedures for epithelial isolation and culturing were recently described (18). In brief, the polyps were placed in a 10-ml test tube with 5 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 mg/l of gentamicin. After ~1 h of gentle shaking, fetal bovine serum (10% vol/vol; Gibco BRL, Life Technologies, 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 each at 110 g) in Ham's F-12 culture medium containing 1% UltraRser G serum substitute (IBI Biotechnics, Savage, MD) and antibiotics as above. The pellet was resuspended in 5 ml of culture medium in a 50-ml tissue culture flask and incubated at 37°C with 5% CO2. During the first 3–4 h, the flask was gently moved every 30 min to reduce attachment of the cell sheets to the bottom of the flask. The medium was changed daily for the first two days and thereafter twice a week by gentle centrifugation (20 g for 2 min) or simple sedimentation for 10 min in a test tube. From the third day, gentamicin was omitted from the medium. Hydrocortisone (5 × 10−6 M) was added 1–4 days before experiments.

Electrophysiology. The electrical parameters were measured in epithelial spheroids transferred to 800 µl of a 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 thermostated chamber and placed on the stage of an inverted microscope (Nikon Diaphot 300 equipped with Hoffman modulation contrast (HMC) optics). Spheroids were kept in position by a Ringer-filled holding pipette (Swemed Laboratory, Billdal, Sweden) with an internal tip diameter of ~25 µm applying gentle suction with a micrometer (IM-6, Narishige). Spheroids were impaled by microelectrodes pulled from filamented borosilicate glass tubes (1 mm OD; Clark Electromedical, Reading, UK) on a horizontal puller (P-97, Sutter Instruments, Novato, CA) and backfilled with 1 M KCl (tip resistance 50–100 MΩ). Impalements were performed perpendicular to the external apical surface of the spheroids with a 3-dimensional piezoelectric micromanipulator (PCS-3200/PZ-301, Burlington, MA) and a 3+1-dimensional hydraulic micromanipulator (MHW-3/MHW-4, Narishige). The microelectrode for PD measurement was connected to a high-impedance electrometer (Duo-773, World Precision Instruments, Sarasota, FL), and the bath was grounded via a Ringer-agar bridge and an Ag-AgCl electrode. The electrical response and a monitor (Sony, J pan) attached via a camera (CCV-931, Videov Euroline, Dortmund, Germany) to the microscope. All experiments were videotaped (AG-7355-E, Panasonic, Osaka, J pan) for later inspection.

Spheroid volume changes and fluid absorption. Fluid absorption was estimated from measurements of the outer diameter of individual spheroids at intervals of 5–20 min. The spheroids were placed in 10-µl droplets of the original culture medium on the bottom of a plastic dish and then covered with paraffin oil (Uvasol, Merck) that had been “saturated” in contact with the culture medium for several days in the incubator. In this way, evaporation and gas exchange from the droplet to the atmosphere during diameter measurements was minimized. Two pictures of each spheroid, focusing on the spheroid perimeter and on the bottom surface of the spheroid, and a picture of the calibration microscale were grabbed and stored in a computer via the video camera with a ×10 objective (Nikon MCI 10, HMC, 0.25 long working distance) and a software-controlled frame grabber (Flashpoint 3.0, Integral Technologies, Indianapolis, IN). From the calibration microscale picture and the diameter at the spheroid perimeter (measured manually on a 20-inch video screen), spheroid volume and surface area were calculated. The fluid transport rate (Jv, in µl·cm−2·h−1) was then calculated from the volume change (∆V/∆t) in a period of measurement (usually 20 min) related to the surface area (A) that was calculated from the average diameter during that period: Jv = ∆V/∆t·A.

From images focusing on the spheroid surface, it was possible in some spheroids to select areas with clearly demarcated cells where surface cell densities were estimated. Simultaneously calculated Jv values per area (described above) then allowed for estimation of Jv values per cell.

When Jv values were studied in spheroids under the influence of amiloride, diphenylamine-2-carboxylate (2-DPC), 5-nitro-2-(3-phenylpropylamino)benzoate (NPPB), or hydrochlorothiazide, the drug was added from stock solutions to prewarmed medium. Then a number of spheroids were added, and individual spheroids were transferred in a droplet to the plastic dish for measurement of volume changes as described above.

For estimates of spheroid epithelial water permeability, fluid absorption was measured (as described above) in response to an inwardly directed 75 mosmol/l gradient. Thus 15 µl of oil-covered culture medium (−300 mosmol/l) containing a spheroid were diluted with 5 µl of water with the use of a micropipette driven by a set of hydraulic and motorized micromanipulators (MM-188 and MO-188, Narishige) connected to a micrometer (IM-6).

Solutions and chemicals. Cell culture media were obtained from ICM Biochemicals (Costa Mesa, CA). 2-DPC and NPPB were gifts from Dr. R. Greger (Albert-Ludwigs-Universität, Freiburg, Germany). All other chemicals were purchased from Sigma (St. Louis, MO). Stock solutions of amiloride were prepared in Ringer solution, whereas stock solutions of 2-DPC, NPPB, and hydrochlorothiazide were prepared in DMSO at 1,000 times the final concentration.

Statistics. All quantitative data are means ± SE from individual observations. Multiple groups were compared with ANOVA. If the ANOVA was significant, the individual values were compared with Student’s t-test or the Mann-Whitney rank sum test. A P value < 0.05 was considered significant.
RESULTS

In agreement with our previous observations (18), the present study demonstrated that protease-released sheets of nasal airway epithelium form fluid-filled spheroids with diameters of 50–800 µm when incubated in a defined serum-free medium. All spheroids showed a fully differentiated monolayered epithelium, with apical cell membranes containing microvilli and cilia facing the outside bath and basolateral membranes pointing toward the central, fluid-filled lumen (18). An intense ciliary activity, confirming the state of high differentiation, was consistently observed.

Spheroid electrophysiology. Application of a range of brief (~1-s) transepithelial current pulses within a short time period (~2 min), with simultaneous measurements of spheroid areas (from video recordings of diameters) and changes in transepithelial PD (∆PD), allowed us to calculate transepithelial current densities (I; in µA/cm²) and to obtain the relationship between I and PD (I-V curves). The I values used resulted in ∆PDs of up to ±50 mV. In this range, linear I-V curves were obtained in both CFSs and NCFSs, showing that the spheroid epithelium behaves as an ohmic resistor. Examples of the I-V relationships obtained from a single NCFS before and after apical application of amiloride (10⁻⁴ M) are shown in Fig. 1. From the I-V curves, Rₚ values were calculated as Rₚ = ∆PD/I. Furthermore, the equivalent Iₚ value was calculated as Iₚ = PD/Rₚ. The electrical parameters obtained in this way from 52 NCFSs (7 donors) and 46 CFSs (6 donors) are presented in Table 1. In compari-

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Values are means ± SE of 52 measurements [7 non-cystic fibrosis (CF) donors] and 46 measurements (6 CF donors). NCFS, non-CF spheroid; CFS, CF spheroid; PD, potential difference; Iₚ, short-circuit current; Rₚ, transepithelial resistance. NS, not significant.

son with the NCFSs, the CFSs exhibited significantly higher values of basolateral side-positive PD (155%; P < 0.001) and Iₚ (170%; P < 0.001), whereas the slightly higher Rₚ of CFSs (115%) was not significantly different from the Rₚ of NCFSs. The addition of amiloride (10⁻⁴ M) to the mucosal bath of 16 NCFSs (5 donors) significantly decreased (by ~90%) both the Iₚ (from 37.4 ± 3.8 to 3.3 ± 0.6 µA/cm²) and the PD (from 15.4 ± 1.4 to 1.7 ± 0.3 mV) but increased the Rₚ (by 40%; from 432 ± 47 to 602 ± 66 Ω·cm²). In 13 CFSs (3 donors), amiloride abolished the PD and Iₚ but increased the Rₚ (by 33%; from 454 ± 71 to 602 ± 98 Ω·cm²).

Spheroid volume changes and fluid absorption. We (18) previously demonstrated that the average size of spheroids appeared to be relatively constant when measured daily for several weeks. This average constancy, however, covered the fact that the size of individual spheroids fluctuated, probably due to inward fluid transport, interrupted by periods of shrinkage due to stretch-induced leakage (18). Initial observations in the present study demonstrated that this also applied when the size of the spheroids was measured within a shorter period of time. Thus in 66 NCFSs (4 donors) incubated in smaller groups in culture medium, measurements of spheroid size with time intervals of 1–1.5 h over a period of 5 h demonstrated that the average diameter only changed minimally and insignificantly (from 242 ± 8 to 251 ± 9 µm). However, in a few cases, it was possible to identify large volume changes in individual spheroids equivalent to fluid absorption of up to 6 µl·cm⁻²·h⁻¹. We therefore started measurements of diameters of individual spheroids at short time intervals (5–20 min) over periods of 3–4 h. It became obvious that the volume of spheroids generally fluctuated, with a long period of increasing volume followed by a brief period of shrinkage. However, in several spheroids, the volume at maximum level as well as at minimum level could be stable for ≥1 h. An example of regular fluctuations is shown in Fig. 2, where the diameter of a single NCFS was measured every 10 min. The diameter increased in two periods (representing fluid absorption) interrupted by a brief reduction in diameter (representing leakage and collapse of the spheroid). In the two fluid absorption periods, an identical increase in diameter of 0.98 µm/min was observed, corresponding to a fluid uptake of 3 µl·cm⁻²·h⁻¹.

Changes in individual spheroid diameters were measured in a series of 34 NCFSs (4 donors) at 20-min
intervals over a period of 4 h during incubation in culture medium. Of these 408 measurements, 56 (14%) demonstrated a decrease in diameter of >10 µm and 195 (48%) demonstrated changes in diameters of less than ±10 µm. In the remaining 157 (38%) 20-min periods, an increase in diameter of >10 µm was observed. Calculation of the increase in volume from the increase in diameter and the average diameter in each of these 157 20-min periods resulted in a J_V value of 3.35 ± 0.12 µl·cm⁻²·h⁻¹. When fluid absorption was calculated from the maximal value of volume increase in each of the 34 NCFSs, a value of 4.42 ± 0.29 µl·cm⁻²·h⁻¹ was obtained. In a similar series of measurements of diameters in 65 CFSs (5 donors), an increase in diameter of >10 µm was observed in a similar fraction of observations as in NCFSs (n = 284 measurements; 40%). The average J_V in these periods was 3.25 ± 0.07 µl·cm⁻²·h⁻¹, whereas the J_V calculated from the maximal value of volume increase in each of the 65 CFSs was 4.35 ± 0.17 µl·cm⁻²·h⁻¹. Thus J_V values were of equal magnitude in NCFSs and CFSs.

In a number of the spheroids incubated under control conditions in culture medium, the J_V per cell was estimated. In these experiments, video-stored images with the focus on the perimeter of a spheroid allowed measurement of total surface area and fluid absorption per square centimeter from recorded diameters and increments in diameter as described above. Simultaneously stored images from spheroids, with the focus on the surface closest to the objective, allowed counting of the cells in a smaller, nearly plane area and thus the number of cells per square centimeter (Fig. 3). The cell densities obtained in this way were ~5 × 10⁶ cells/cm². The calculated average J_V per cell in periods where spheroid diameters increased >10 µm/20 min was 6.4 ± 0.2 pl/h in NCFSs (n = 19 measurements; 3 donors). A similar single-cell absorption rate of 6.6 ± 0.3 pl/h was obtained in CFSs (n = 18 measurements; 3 donors), again emphasizing that fluid absorption in NCFSs and CFSs were of equal magnitude.

The volume change of a spheroid during an increase in diameter from 410 to 460 µm over a period of 30 min is shown in Fig. 4. This illustrates the problem of calculating absolute fluid absorption per square centimeter because the epithelial cells are stretched as the result of spheroid fluid absorption. Thus fluid absorption in this spheroid calculated in relation to surface area at the initial diameter (410 µm) was 5.49 µl·cm⁻²·h⁻¹, whereas the calculated J_V in relation to surface area at the average diameter (435 µm) was 4.88 µl·cm⁻²·h⁻¹. To investigate a coupling between water flux and Na⁺ absorption, spheroids were incubated in the presence of amiloride (100 µM) in the bathing medium. This resulted in a decrease in J_V per cell because the epithelial cells are stretched as the result of spheroid fluid absorption. Thus fluid absorption in this spheroid calculated in relation to surface area at the initial diameter (410 µm) was 5.49 µl·cm⁻²·h⁻¹, whereas the calculated J_V in relation to surface area at the average diameter (435 µm) was 4.88 µl·cm⁻²·h⁻¹.
resulted in \( J_V \) values considerably lower than those under control conditions. Consequently, estimates of fluid absorption were based on longer periods of diameter measurements (40–200 min) than the 20-min periods used for measurements of maximal fluid absorption under control conditions. The amiloride-insensitive fluid absorption was 0.51 ± 0.07 µl·cm\(^{-2}\)·h\(^{-1}\) in NCFSs (n = 23 measurements; 5 donors). This value is significantly lower than the fluid absorption of 1.27 ± 0.08 µl·cm\(^{-2}\)·h\(^{-1}\) observed in amiloride-treated CFSs (P < 0.001; n = 44 measurements; 5 donors). This difference in fluid absorption may be related to a defective Cl\(^-\)secretion in CFSs due to a missing CF transmembrane conductance regulator (CFTR) function (1).

We (18) previously demonstrated that apical cell membrane Cl\(^-\)-channel blockade by 2-DPC eliminated the amiloride-insensitive PD in NCFSs. We therefore tested the effects on NCFS fluid transport of the Cl\(^-\)-channel inhibitors 2-DPC (250 µM) and NPPB (100 µM) in combination with amiloride. With these inhibitors, we obtained values of \( J_V \) (2-DPC plus amiloride: 1.11 ± 0.12 µl·cm\(^{-2}\)·h\(^{-1}\), n = 8 measurements, 2 donors; NPPB plus amiloride: 1.11 ± 0.12 µl·cm\(^{-2}\)·h\(^{-1}\), n = 7 measurements, 2 donors) significantly higher than those obtained with amiloride alone (P < 0.001; Fig. 5). This suggests that amiloride-insensitive water absorption in NCFSs is counterbalanced by an amiloride-induced Cl\(^-\) and water secretion. Interestingly, the \( J_V \) values in NCFSs treated with amiloride and Cl\(^-\)-channel blockers were not significantly different from that in CFSs treated with amiloride only (Fig. 5).

In CFSs, addition of amiloride abolished the \( I_w \) (and the PD; Table 1). The presence of a significant amiloride-insensitive residual fluid absorption in CFSs (as well as in NCFSs treated with amiloride or amiloride plus Cl\(^-\)-channel inhibitors; see above) therefore suggested that an electroneutral absorptive process was responsible for this fluid absorption. The nature of such a transport mechanism is unknown, but a plausible candidate could be an apical membrane Na\(^+\)/Cl\(^-\) cotransporter (19). We therefore tested the effects of the specific Na\(^+\)/Cl\(^-\) cotransport inhibitor hydrochlorothiazide (22). Figure 5 shows that in 29 CFs exposed to mucosal amiloride (100 µM) and hydrochlorothiazide (100 µM), fluid absorption (0.85 ± 0.07 µl·cm\(^{-2}\)·h\(^{-1}\)) was significantly lower than in CFSs treated only with amiloride (1.27 ± 0.08 µl·cm\(^{-2}\)·h\(^{-1}\); P < 0.001). Treatment of NCFSs in the same way with amiloride plus hydrochlorothiazide also resulted in significantly lower fluid absorption (0.15 ± 0.04 µl·cm\(^{-2}\)·h\(^{-1}\); n = 17 measurements) compared with that obtained with amiloride only (0.51 ± 0.07 µl·cm\(^{-2}\)·h\(^{-1}\); P < 0.001). Thus in both CFSs and NCFSs, an electroneutral, hydrochlorothiazide-sensitive absorptive mechanism is responsible for a fluid absorption of ~0.4 µl·cm\(^{-2}\)·h\(^{-1}\), which is equal to ~10% of the control \( J_V \) value.

In a series of experiments, we estimated the osmotic water permeability of the spheroid airway epithelium. Single spheroids were placed in a 15-µl droplet of incubation medium (osmolarity = 300 mosmol/l) covered with paraffin oil in the thermostated chamber on the microscope stage. The diameter of the spheroid was videorecorded before and after the addition of 5 µl of water to the droplet [resulting in a lowering droplet (apical) osmolarity of ~75 mosmol/l]. In response to this imposed transepithelial osmotic gradient (corresponding to an equivalent ΔP of 1.91 atm), spheroid volume increased much faster than during spontaneous fluid absorption. From the fast increase in volume during the first 5 min (in those spheroids that remained intact during the increased fluid absorption), we calculated \( J_V \) values of 21.14 ± 1.53 µl·cm\(^{-2}\)·h\(^{-1}\) in NCFSs (n = 22 measurements; 3 donors) and 21.82 ± 1.96 µl·cm\(^{-2}\)·h\(^{-1}\) in CFSs (n = 10 measurements; 2 donors). On the basis of these equal \( J_V \) values, we calculated a corresponding hydraulic conductivity (\( L_P = J_V \sqrt{\Delta P} \)) of ~27 × 10\(^{-7}\) cm·s\(^{-1}\)·atm\(^{-1}\) and a filtration (osmotic) permeability of ~45 × 10\(^{-4}\) cm/s (\( P_f = L_P RT/V_w \), where \( R \) is the gas constant, \( T \) the absolute temperature in K, and \( V_w \) the partial molar volume of water) in both NCFSs and CFSs.

**DISCUSSION**

We (18) recently introduced a spheroid preparation for the study of ion transport in CF and non-CF airway epithelia and demonstrated that the basolateral side (inside)-positive transepithelial PD of this preparation during the influence of various agonists and ion channel blockers mimics that of the native tissue and conventionally cultured airway cells. The values obtained in the present study are in agreement with those previously obtained (18). The results from passage of current pulses show that the epithelia of both NCFSs and CFSs behave as ohmic resistors, i.e., that the instantaneous I-V curves were linear in the range of at least ±150 µA/cm\(^2\) (Fig. 1). This is in agreement with previous reports (6, 24). Calculation of \( R_t \) values from the slope of the I-V curve demonstrated that the \( R_t \) of...
the spheroid airway epithelium is relatively high (non-CF control \( R_t = 400-450 \ \Omega \cdot \text{cm}^2 \); Table 1). In freshly isolated preparations of nasal airway epithelium, considerably lower \( R_t \) values (50-100 \( \Omega \cdot \text{cm}^2 \)) have been observed (6, 15, 27), whereas high \( R_t \) values (200-1,000 \( \Omega \cdot \text{cm}^2 \)) are generally observed in cell culture preparations of nasal and other airway epithelia (14, 24, 26, 27). As previously emphasized, the present spheroid-type preparation of airway epithelium is not a primary culture but an explant of fully differentiated epithelium. However, like primary cultures, the spheroids were incubated in culture medium during preparation and storage. It seems that incubation in culture medium may result in a decrease in the conductance of the paracellular pathway; i.e., the epithelium is transformed from a low-resistance epithelium in the direction of a high-resistance epithelium (28). In the present spheroid preparation, the conductance of the cellular pathway is not insignificant compared with the conductance of the paracellular (shunt) pathway. This interpretation is supported by the observations 1) that closure of apical Na\(^+\) channels by amiloride consistently increased \( R_t \) in NCFSs (by \( \approx 40\% \)) and CFSs (by \( \approx 33\% \)) and 2) that the \( R_t \) in CFSs (lacking the cellular conductance from CFTR Cl\(^-\) channels) was higher than that in NCFSs (the difference was not significant due to the scatter in \( R_t \) values). Similarly, slightly higher or equal \( R_t \) values in CF airway epithelium compared with those in non-CF epithelia have been previously reported (6, 14, 16, 24).

The \( I_{sc} \) in upper airway epithelia is primarily a measure of amiloride-sensitive Na\(^+\) absorption (3, 5, 6). In accordance with this, we observed that apical amiloride decreased \( I_{sc} \) (measured as equivalent \( I_{sc} = PD/R_t \)) by 91% in NCFSs and 100% in CFSs. Also, the observation that \( I_{sc} \) in untreated preparations was significantly higher in CFSs than in NCFSs (Table 1) is in accordance with a reported increase in epithelial capacity for Na\(^+\) absorption in CF airway epithelia caused by upregulation of apical membrane Na\(^+\)-channel permeability (4–6). The residual, amiloride-insensitive \( I_{sc} \) (and PD) in NCFSs may at least partly be accounted for by Cl\(^-\) secretion through apical (CFTR) Cl\(^-\) channels triggered by amiloride-induced apical membrane hyperpolarization (3, 26) as demonstrated by its sensitivity to the Cl\(^-\)-channel inhibitor 2-DPC in NCFSs (18). However, it should be emphasized that in the present preparation of NCFSs, such a Cl\(^-\) secretion is rather small because amiloride inhibited \( I_{sc} \) (and PD) by \( \approx 90\% \). This could be due to a small driving force for Cl\(^-\) movement across the apical membrane (26) or to a low activation of Cl\(^-\) channels. In CFSs, the lack of a residual \( I_{sc} \) after amiloride may obviously be related to the lack of CFTR Cl\(^-\) channels.

We (18) previously observed that the size of individual spheroids fluctuated around an average value that appeared to be almost constant for several weeks. It was suggested that these volume fluctuations reflected periods of inward fluid transport interrupted by episodes of shrinkage caused by pressure- and/or stretch-induced openings of tight junctions. We further-more suggested that the fluid absorption was driven by active inward transport of Na\(^+\). The present study confirms these suggestions. When maximal rates of volume increase were related to the simultaneous average spheroid surface, \( J_V \) values of equal magnitude were obtained in NCFSs and CFSs. Similar magnitudes of \( J_V \) values by non-CF airway epithelial preparations have been reported in some studies (2, 14, 23, 29, 30), whereas other studies (12, 16, 20, 21) have demonstrated lower values. Most frequently, increased levels of \( J_V \) values are observed in CF airway epithelial preparations (14, 16, 30). Although decreased levels have also been reported in CF preparations (29), our finding of equal \( J_V \) values in NCFSs and CFSs is therefore at variance with some previous studies. The values of \( J_V \) in the present study were calculated in relation to surface area at a time when the spheroids had already been stretched to some degree. Thus absolute absorption rates may be underestimated considerably. This is also suggested from the observed surface cell density of \( \approx 5 \times 10^3 \) cells/cm\(^2\) during maximal spheroid fluid absorption compared with reported cell densities of up to \( \approx 45 \times 10^3 \) cells/cm\(^2\) in unstretched airway epithelium (17). These considerations may suggest that fluid absorption in the present explant preparation may be considerably higher than in conventional primary cultures if it is related to unstretched epithelial surface. However, they do not explain the similarity in fluid absorption in CFSs and NCFSs. Thus equal \( J_V \) values in the two types of spheroids were found not only when related to surface area but also when fluid absorption per cell was calculated. Stretching of the spheroid surface area may, in a similar way and to a similar degree, lead to an underestimation of the above-mentioned \( I_{sc} \) values. For comparison of ion and water transport capacities (see below), corrections for surface stretching are therefore not necessary.

The mechanism of coupling between net salt and water transport in airway epithelia is still unknown. Thus it is not known whether a coupling is at the intraepithelial or the transepithelial level. Studies on fluid transport across varying types of airway epithelial preparations demonstrate no general agreement that water follows net solute movements in the absence of transepithelial solute concentration gradients (16, 20, 29). A study (23), however, has demonstrated parallel changes in net solute and water movements that are compatible with an intraepithelial, isosmotic coupling. However, in the present spheroid preparation, water uptake probably has to follow net salt uptake in isosmotic proportions. The inside (basolateral) volume of spheroids is small. Consequently, if water does not follow net salt absorption at the intraepithelial level, salt absorption will tend to increase the inside osmolarity, and provided the epithelial osmotic water permeability is sufficiently high, water will follow passively. Indications of high water permeability have been presented in intact airway preparations (8) and in primary cultures of human airway epithelium (7, 9, 12, 16). The osmotic water permeabilities observed in the present...
study demonstrate identical values in CF and non-CF epithelia ($L_p = \sim 27 \times 10^{-7} \text{ cm s}^{-1} \cdot \text{atm}^{-1}$). These values are as high as those measured in the highly water-permeable epithelia in the mammalian small intestine, gallbladder, and plexus choledochus (13). They even have to be regarded as minimum values due to the likely presence of unstirred layers and to the fact that the osmotic water flows were related to a slightly stretched surface area. Furthermore, a comparison of calculated net ion transport rates with $J_V$ values indicates that absorbed fluid may be close to isosmotic with the bathing medium. With the observations that the equivalent $I_{oc}$ of NCFSSs represents Na$^+$ absorption, that anions follow passively, and that water follows NaCl isosmotically, a fluid absorption of $\sim 7 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$ was calculated, a value close to observed $J_V$ values.

The ion transport mechanisms reflecting the $J_V$ values under open-circuit conditions may be explained as follows: in untreated NCFSSs, transcellular, active Na$^+$ absorption (of a size reflected by the $I_{oc}$) is followed by passive anion absorption via the paracellular pathway and/or a cellular pathway (basolateral and cAMP-stimulated apical membrane Cl$^-$ channels; 21, 23, 25). Water follows the resulting net NaCl absorption. In CFSSs, NaCl and water absorption are expected to be higher than in NCFSSs due to upregulation of apical membrane Na$^+$-channel conductance and transepithelial Na$^+$ transport capacity (reflected by an increase in $I_{oc}$) (4). However, the present observed $J_V$ values are not higher in CFSSs than in NCFSSs, which indicate that the actual Na$^+$ absorption during open-circuit conditions is not increased. The reason for this may be that under open-circuit conditions, the apical membrane Na$^+$ uptake is slowed down due to a smaller driving force (the PD across the apical membrane is considerably smaller in CF airway cells compared with a cellular PD of approximately $\sim 25 \text{ mV}$ across the apical membrane in non-CF cells (5, 6, 24)). Furthermore, the lack of apical membrane CFTR Cl$^-$ channels excludes the possibility of coupled, passive transport of the counterion Cl$^-$ via the cellular pathway.

The effects of amiloride in the bathing medium also disclose relationships between ion transport and water absorption in the spheroid preparations. Amiloride treatment of NCFSSs resulted in a considerably lower fluid absorption ($0.46 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) than in nontreated NCFSSs (4.4 $\mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$). Amiloride reduces fluid absorption in CFSSs but to a significantly smaller degree (from 4.5 to $1.27 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$). Other studies have also shown that amiloride reduces fluid absorption in non-CF (2, 14, 20, 23, 29) and CF (14, 29) epithelia. In amiloride-treated CFSSs, Na$^+$ absorption via apical Na$^+$ channels is abolished and cellular Cl$^-$ secretion (via CFTR Cl$^-$ channels) is not possible. This situation is characterized by abolition of transepithelial PD and $I_{oc}$. The fact that spheroid fluid uptake did not vanish in response to amiloride in either CFSSs or NCFSSs suggests that a nonelectrogenic NaCl absorption could be responsible for the amiloride-insensitive fluid absorption. In rabbit nasal airway epithelium, a small, nonelectrogenic, and hydrochlorothiazide-sensitive Na$^+$ absorption was previously demonstrated (19). Apical application of hydrochlorothiazide (100 $\mu M$) reduced fluid absorption to the same extent ($0.3$–$0.4 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) in amiloride-treated preparations of both CFSSs and NCFSSs, suggesting the presence of an electroneutral Na$^+$/Cl$^-$ cotransporter (22) in the apical cell membrane.

The difference in fluid absorption ($\sim 0.8 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) between amiloride-treated CFSSs and NCFSSs may suggest the presence in NCFSSs of a NaCl secretion superimposed on the residual amiloride-resistant NaCl absorption. Thus in NCFSSs, the inhibition of apical Na$^+$ channels (and electrogenic Na$^+$ absorption) by amiloride is expected to hyperpolarize the apical cell membrane and thereby induce a small secondary active cellular Cl$^-$ secretion via apical membrane (CFTR) Cl$^-$ channels (and a basolateral membrane Na$^+$/K$^+$/2Cl$^-$ cotransporter). In this situation, a passive, paracellular Na$^+$ movement may follow the cellular Cl$^-$ secretion, and a component of fluid movement is expected to occur in the secretory direction. This interpretation is supported by the observation that apical applications of Cl$^-$-channel inhibitors (2-DPC or NPPB) to amiloride-treated NCFSSs resulted in a significantly higher $J_V$ ($1.11 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) with either 2-DPC or NPPB) than in the absence of Cl$^-$-channel inhibitors ($0.46 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$). Interestingly, these values are not different from the $J_V$ values in amiloride-treated CFSSs ($1.27 \mu l \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) lacking the possibility of Cl$^-$ secretion through CFTR Cl$^-$ channels.

The present study as well as other recent studies in primary cultures of airway cells (23, 29) suggests that a part of the Cl$^-$ absorption in non-CF airway epithelium under open-circuit conditions may take a cellular route via apical and basolateral membrane Cl$^-$ channels. This seems to be at variance with previous suggestions from studies (3, 5, 19) in freshly isolated natural airway epithelia where the pathway for passive Cl$^-$ absorption was interpreted to be paracellular. This discrepancy may be related to the above-mentioned fact that natural epithelia are low-resistance epithelia, whereas primary cultured airway cell layers (and the present spheroid explant) are high-resistance epithelia. The difference in resistance probably reflects a difference in the resistance of the paracellular tight junction pathway (10). In low-resistance epithelia, this pathway serves as an intraepithelial shunt that hyperpolarizes the apical cell membrane (as under short-circuit conditions or by blocking apical Na$^+$ channels with amiloride), thereby favoring apical Cl$^-$ exit and Cl$^-$ secretion instead of Cl$^-$ uptake. Under high-resistance conditions (with less shunting), the apical Cl$^-$ gradient may be unfavorable for absorption, and, simultaneously, the relative contribution to Cl$^-$ absorption from paracellular passage may be small due to a lower Cl$^-$ permeability.

It should be emphasized that even in the absence of knowledge about coupling mechanisms between salt and water flows, we may expect that absorption in the in vivo upper airways has to be isosmotic because 1) the volume of the apical periciliary liquid layer is small, 2) the epithelial osmotic water permeability is high, and
3) the time of contact between surface liquid and epithelium is long due to a relatively slow mucociliary clearance. It is therefore unlikely that in vivo airway epithelia can maintain transepithelial osmotic gradients. Our results with spheroids being exposed to a sudden reduction of tonicity (by 75 mosM) of the apical solution support this conclusion. Thus osmotically driven fluid absorption was approximately five times larger than the spontaneous J_v.

In conclusion, the present study on spheroid preparations of airway surface epithelium demonstrates that 1) unstimulated J_v values under open-circuit conditions are equal in CFSSs and NCFSSs; 2) water absorption is closely related to net NaCl absorption as determined by the concerted function of amiloride-sensitive Na^+ absorption, Cl^- secretion, and an electroneutral NaCl cotransport mechanism; and 3) that the high water permeability of spheroids makes it unlikely that the human airway epithelium can maintain osmotic gradients.

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