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Original article

Altered ion transport and responsiveness to methacholine and hyperosmolarity in air interface-cultured guinea-pig tracheal epithelium

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Abstract

Introduction: Challenge of guinea-pig tracheal epithelium with hyperosmolar solution alters ion transport and evokes the release of epithelium-derived relaxing factor (EpDRF). Cultured tracheal epithelial cells (CE) offer the potential to examine biochemical pathways related to EpDRF release, but whether the bioelectric properties and responses of fresh, adherent epithelial cells (FE) are modeled by CE has not been established. **Methods:** Tracheal epithelial cells grown in air-interface culture and fresh tracheal segments were mounted in Ussing chambers to determine short circuit current (I_{sc}) and transepithelial resistance (R_t) and to compare responses to transport inhibitors, methacholine and hyperosmolarity. **Results:** Significant differences in basal I_{sc} and R_t between FE and CE were observed (I_{sc}, 41.3±3.5 and 8.5±0.8 μA/cm^2, $P<0.05$; R_t, 106±7 and 422±4 Ω cm^2, $P<0.05$; respectively); basal spontaneous potential difference values were not different (4.2±0.3 and 3.4±0.3 mV, respectively). Amiloride (mucosal, 3×10^{-5} M), bumetanide (basolateral, 10^{-5} M) and ouabain (basolateral, 10^{-5} M) reduced I_{sc} equally in FE and CE. In contrast, NPPB (10^{-5} M) in the presence of amiloride had a differential effect, decreasing I_{sc} by 11% in FE and 71% in CE ($P<0.05$). Iberiotoxin (basolateral, 10^{-7} M) was without effect in either preparation. In FE, serosal methacholine (3×10^{-5} M) elicited an NPPB-insensitive monotonic increase in I_{sc}, but in CE caused a large, transient, NPPB-inhibitable increase which was followed by an NPPB-resistant plateau. Addition of apical D-mannitol (0.3–267 mosM) to increase osmolarity decreased I_{sc} in FE, whereas in CE D-mannitol initially increased (0.3–84.3 mosM) and then decreased (84.3–267 mosM) I_{sc}. **Discussion:** Cell culture causes substantial changes in the bioelectric and pharmacological properties of respiratory epithelium. Caution should be exercised when using CE as a substitute for FE in studies of ion transport- and cell volume-dependent processes.

Keywords: Airway; Bioelectric responses; Guinea pig; Epithelium; Cell culture effects; Hyperosmolarity; D-Mannitol; Methacholine; Methods; Ussing chamber

1. Introduction

The epithelium in the respiratory tract has many complex biological roles. It serves as a protective barrier that limits the movement of inhaled chemicals, particulates and other agents into the body and maintains the composition of the airway surface liquid (ASL) through regulated transepithelial ion transport, thereby allowing efficient propulsion of substances up the mucociliary escalator by ciliated epithelial cells. In addition, the epithelium metabolizes drugs, neurotransmitters and inflammatory mediators (Goldie & Hay, 1997) and secretes an array of inflammatory mediators (Churchill et al., 1989; Churchill, Friedman, Schleimer, & Proud, 1992; Diamond, Legarda, & Ryan, 2000; Ge et al., 2004; Guo et al., 1997; Holtzman, Ferdman, Bohrer, & Turk, 1991; Kwon et al., 1994; Lilly et al., 1997; Marini, Vittori, Hollemborg, & Mattoli, 1992; Sousa et al., 1994; Watkins, Garlepp, & Thompson, 1997). Epithelial cells are involved in host

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defense, innate immunity (Bals & Hiemtra, 2004) and resolution of inflammation (Sexton, Al-Rabia, Blaylock, & Walsh, 2004). In addition, the epithelium may be involved in airway remodeling observed in asthma through release of growth factors such as transforming growth factor-β1 (Kumar, Herbert, & Foster, 2004).

The respiratory epithelium is also involved in the regulation of the reactivity of airway smooth muscle through the release of epithelium-derived relaxing factor (EpDRF)(Fedan, Hay, Farmer, & Raeburn, 1988; Flavahan, Aarhus, Rimele, & Vanhoutte, 1985; Folkerts & Nijkamp, 1998; Goldie & Hay, 1997), which can diffuse to the smooth muscle and inhibit contraction or induce relaxation. EpDRF is released following exposure of the epithelium to hyperosmolar solution (Fedan et al., 1999, 2000; Munakata, Mitzner, & Menkes, 1988). The significance of this phenomenon is that the ASL is thought to become hyperosmolar during the hyperventilation that occurs during exercise, causing bronchodilation in normal individuals but obstruction in asthmatic patients (Anderson and Daviskas, 1992). The ability of inhaled D-mannitol (D-M) to elicit obstruction in asthmatics is thought to be initiated by a rise in ASL osmolarity (Anderson and Brannan, 2003). Release of EpDRF following hyperosmolar challenge is associated with bioelectric events in the epithelium that are linked to changes in $Na^+$and Cl^- transport (Dortch-Carnes, Van-Scott, & Fedan, 1999; Fedan et al., 1999; Wu, Johnston, Rengasamy, Van Scott, & Fedan, 2004). The identity of EpDRF has not been established unequivocally, but evidence has been obtained to suggest that it resembles carbon monoxide in some respects (Fedan, Dowdy, Johnston, & Van Scott, 2004; Fedan, Dowdy, Van Scott, Wu, & Johnston, 2004).

Elucidation of the mechanisms involved in EpDRF release could be facilitated by the use of cultured epithelial cells. Previous work on EpDRF has been performed using the adherent epithelium in the guinea-pig trachea. Confirmation that the physiological and pharmacological properties of this tissue are retained in cell culture is particularly required before signaling pathways are explored biochemically. Therefore, in this report we compared the bioelectric properties and responses to muscarinic stimulation and hyperosmolar solutions in guinea-pig air-interface-cultured epithelial cells (CE) and fresh cells (FE) attached to the tracheal wall. Our results indicate that basal bioelectric properties and responses to hyperosmolar challenge and methacholine are altered in the cultured cells.

2. Methods

2.1. Animals

These studies were conducted in facilities accredited fully by the Association for the Assessment and Accreditation of Laboratory Animal Care International and were approved by the institutional Animal Care and Use Committee. Male guinea pigs (300–350 g; Crl:HA) from Charles River (Wilmington, MA), monitored free of endogenous viral pathogens, parasites, and bacteria, were used in all experiments. The animals were acclimated before use and were housed in filtered ventilated cages on Alpha-Dri virgin cellulose chips and hardwood Beta-chips as bedding, provided HEPA-filtered air, Teklad 7006 diet and tap water *ad libitum*, under controlled light cycle (12 h light) and temperature (22–25 °C) conditions. The animals were anesthetized with sodium pentobarbital (65 mg/kg, i.p.) and sacrificed by thoracotomy and bleeding before removing the trachea.

2.2. Preparation of CE

The cells were isolated from guinea-pig tracheal epithelium and cultured using published methods (Adler, Cheng, & Kim, 1990; Robison & Kim, 1994) for air-interface culture, with modifications. After anesthesia, a 4-cm length section of trachea was removed under sterile conditions and placed in Hank's balanced salt solution (HBSS) containing amphotericin B (1 μg/ml), gentamycin (100 μg/ml) and nystatin (40 U/ml). The trachea was cleaned of extraneous tissue under a dissection microscope, cut open longitudinally through the smooth muscle band, and incubated with 0.2% protease in minimum essential medium (MEM) at 37 °C in 95% air–5% CO_2 for 1 h. Epithelial cells were gently scraped off with a scalpel, pooled from different animals, suspended with gentle trituration in MEM containing 10% fetal bovine serum (FBS) and 1 mg/ml DNase, and centrifuged for 5 min at 250 *g* at room temperature. The supernatant was discarded and the pellet was suspended in 10 ml MEM containing 10% FBS. Cells were suspended and centrifuged for a second time in 10 ml MEM containing 10% FBS. Then, cells were filtered through a 70 μm filter and centrifuged a third time. Finally, cells were suspended in 1 ml PC-1 medium containing L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and retinoic acid (10^{-7} M). (A significant improvement to the published methods was the addition of retinoic acid to the culture medium. Retinoic acid increased the pseudostratification of the epithelial cells, and led to an increase in the prevalence of ciliated cells.) Cells were plated on permeable matrix, Costar 7 12-mm Snapwells (Corning, Inc.), 10^6 cells per well. All culture wells had been coated previously with type-1 rat-tail collagen. A stock solution (50 μg/ml) of collagen was prepared in 0.02 N glacial acetic acid. The solution had been sterilized by passage through a 0.22 μm filter and added to the wells in an amount of 60 μg/cm^2. The wells were incubated at room temperature overnight and were allowed to dry. The coated wells were rinsed with HBSS and PC-1 media before the cells were seeded into the wells.

The cells were initially cultured in immersion with PC-1 medium containing L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and retinoic acid (10^{-7} M) at 37 °C in 95% air–5% CO_2 to allow adherence to the collagen-coated matrix. After 24 h, non-adherent cells were rinsed away with PC-1 medium, and the cells were cultured in air-interface at 37 °C in 95% air–5% CO_2. The PC-1 medium in the bottom chamber was changed daily.

2.3. Monitoring CE confluence and transepithelial resistance (R_t)

The cells were observed daily using a phase-contrast microscope (Zeiss Axiovert 100TV). Upon reaching confluence (2 to 3 days), R_t was measured daily by placing the Snapwell into an Endohm−24 Snap which was connected to a current-passing electrometer (EVOM, World Precision Instruments, Inc.).

2.4. Electrophysiological studies of CE

For assessment of bioelectric properties, confluent cells were used within 24 h of reaching R_t of 1000 Ω or greater. The Snapwells were mounted between the hemi-chambers of a Ussing apparatus and perfused continuously with modified Krebs–Henseleit solution (MHKS). A pair of EKV cartridge electrodes (World Precision Instruments, Inc.), each containing 4% agar in saline, were placed 3 mm from the orifice to detect V_t; a pair of EKC cartridge electrodes (World Precision Instruments, Inc.), each containing 4% agar in saline, were placed within 2 cm of the orifice to deliver a calibrated voltage for determination of R_t. Both EKV and EKC electrodes were connected to a voltage/current clamp amplifier (DVC 3000, World Precision Instruments, Inc). A cell-free Snapwell was first loaded into the Ussing chamber while measuring electrode potential difference and fluid resistance; both were compensated for before loading the Snapwells containing the cells into the chamber. V_t was monitored under open-circuit conditions, and it usually reached stability within a 3-h equilibration period. Thereafter, V_t was clamped at 0 mV while continuously measuring short circuit current (I_{sc}). Voltage pulses (1 mV square waves sustained for 5 s) were delivered every 50 s to yield a current response for calculation of R_t from Ohm's law. The data were logged on a strip-chart recorder and into data acquisition software, from which the results were quantified. Both apical and basolateral baths were washed at 15-min intervals during the equilibration period.

2.5. CE morphology

For electron microscopic examination of CE, the cells adherent to Snapwells were washed twice with phosphate-buffered saline, fixed for 60 min at room temperature with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), and post-fixed for 1 h at 4 °C with 1% osmium tetroxide in 0.1 M sodium phosphate buffer (pH 7.2). The cells were then stained with 1% uranyl acetate in 0.05 M sodium maleate (pH 5.2), dehydrated through serial concentrations of ethanol, infiltrated, and embedded with Epon 812 (Electron Microscopy Sciences). Semi-thin sections (0.5 μm) were cut with glass knives and stained with toluidine blue for examination with a light microscope. Thin sections of specific areas were obtained with a diamond knife, contrasted with uranyl acetate and lead citrate, and examined with an electron microscope (JEOL 100s).

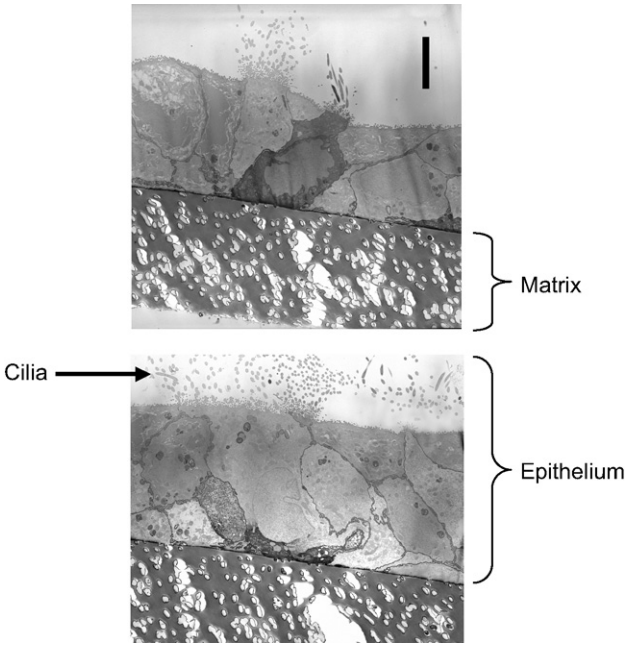

Fig. 1. Representative transmission electron micrographs of CE grown in air-interface culture on Snapwells. Bar = 5 μm.

Table 1
Basal bioelectric parameters for CE and FE

Cell type	SPD (mV)[a]	I_{sc} (μA/cm^2)	R_t (Ω·cm^2)
CE	3.4±0.3	8.5±0.8	422±41
FE	4.2±0.3	41.3±3.5[b]	106±7[b]

[a]Spontaneous potential difference (SPD) calculated from Ohm's law. CE, $n=12$. FE, $n=19$. [b]CE *vs.* FE, $P<0.05$.

2.6. Preparation of FE from guinea-pig trachea for assessment of bioelectric properties

A 4-cm segment of the trachea was removed from anesthetized guinea pigs (500–550 g), placed into MKHS and cleaned. The segment was cut open through the smooth muscle band, mounted in a CHM8 Ussing chamber (World Precision Instruments, Inc.), and perfused with MKHS bubbled with 95% O_2–5% CO_2 at 37 °C. Mucosal and serosal baths were washed at 15-min intervals. Voltage and current electrodes were arranged as described above. When V_t reached a stable value after *ca.* 3 h, the cells were voltage-clamped at 0 mV, and data were recorded as described above.

2.7. Effects of ion transport inhibitors, methacholine (MCh), and hyperosmolar solution

The effects of the following ion transport inhibitors were examined after the 3-h equilibration period: amiloride (apical; 3×10^{-5} M), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; apical, 10^{-5} M); bumetanide (serosal; 10^{-5} M); ouabain (serosal, 10^{-5} M); iberiotoxin (serosal, 10^{-5} M). For study of the responses to hyperosmolar challenge with step-wise increasing cumulative concentrations of D-M, MCh, in a concentration (3×10^{-7} M) that is the ~EC50 for contraction, was first added to the basolateral bath in order to mimic conditions that have been used in EpDRF studies in perfused trachea (Fedan et al., 1999). An equal volume of MKHS was added simultaneously to the basolateral bath to equalize hydrostatic pressure across the segment.

2.8. Solutions and drugs

MKHS contained: NaCl 113.0 mM, KCl 4.8 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25.0 mM and glucose 5.7 mM, pH 7.4 (37 °C); and was gassed with 95% O_2–5% CO_2. The osmolarity of MKHS was 281±5 mosM. HBSS and PC-1 media were from Cambrex Biosciences; the remaining drugs and reagents were from Sigma-Aldrich.

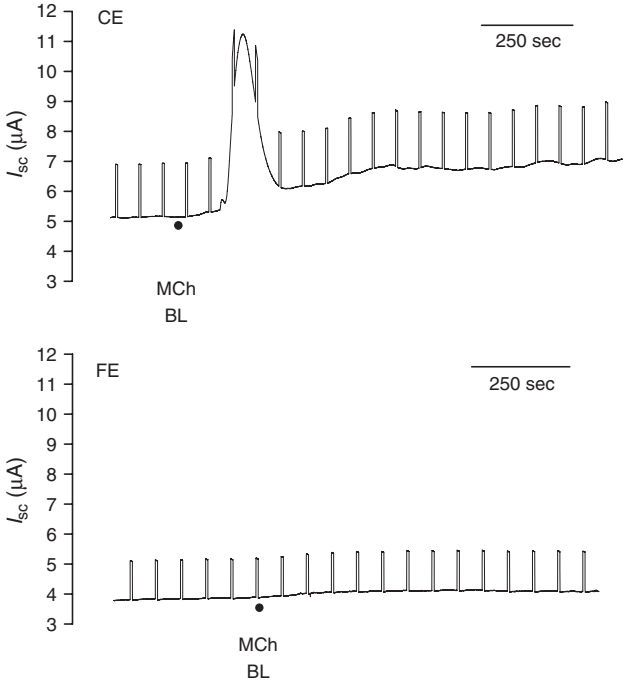

Fig. 2. Representative tracings showing responses of CE (top) and FE (bottom) to the basolateral (BL) addition of 3×10^{-7} M MCh. Shown in the figure are I_{sc} values that are not corrected for surface area; values normalized for surface area are shown in Fig. 3. The deflections shown in this and other like figures are the current responses resulting from the 1 mV voltage pulses of 5 s duration that were delivered every 50 s.

2.9. Analysis of results

The results are presented as mean±S.E.; n is the number of separate experiments. The effects of agents on I_{sc} and R_t values of CE and FE were compared using Student's t-test or the Mann–Whitney rank sum test for paired samples. Differences between CE and FE values were compared using Student's t-test for unpaired samples. $P<0.05$ was considered significant.

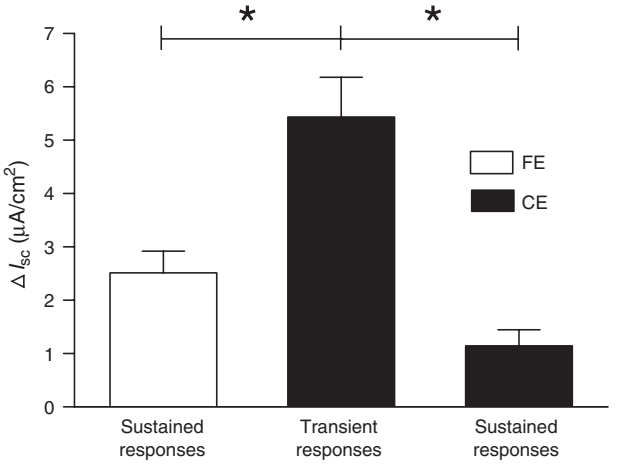

Fig. 3. Responses of FE and CE to basolaterally-added MCh (3×10^{-7} M). "Sustained responses" were quantified at the point where the responses had become stable; "transient responses" were quantified as the peak value of the response. $n=19$ and 9 for FE and CE, respectively. *Transient responses significantly different from sustained responses of FE (non-paired analysis) or sustained responses of CE (paired analysis).

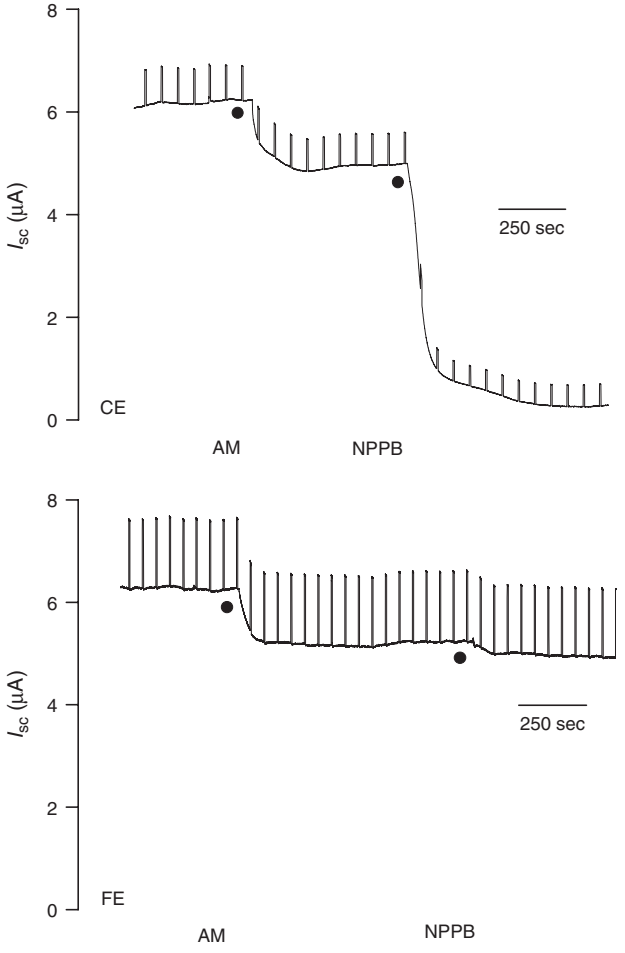

Fig. 4. Effects of apical amiloride (3×10^{-5} M) and apical NPPB (3×10^{-5} M) on I_{sc} of CE (top panel) and FE (bottom panel). The results shown here are not corrected for surface area, which is summarized in Fig. 5. These results are representative of $n=9$ and 8 separate experiments for FE and CE, respectively.

3. Results

3.1. Morphology of CE

A measurable R_t was detected on the second day of culture (the first day of the air interface); it reached its peak by the third day, and it was maintained for several days (data not shown). Electron

Table 2
Effects of ion transport blockers on R_t[a] in FE and CE

	FE		CE	
Blocker	Before	After	Before	After
Amiloride	88.5±5.4	89.1±6.0 (7)	483.2±62.6	466.4±62.8 (7)
NPPB[b]	89.1±6.0	98.7±8.3[c] (8)	558.6±88.5	590.6±101.7 (10)
Bumetanide	101.9±17.7	98.8±17.8 (6)	450.8±90.8	612.2±129.3 (6)
Ouabain	87.8±9.4	83.6±9.4 (10)	741.6±132.6	722.9±119.8 (4)
Iberiotoxin	102.2±23.2	102.0±23.5 (4)	287.2±8.3	285.2±7.3 (6)

[a]Ω·cm². [b]Values shown were obtained from experiments in which amiloride was present. [c]Before *vs.* after, $P<0.05$. n values are shown in parentheses.

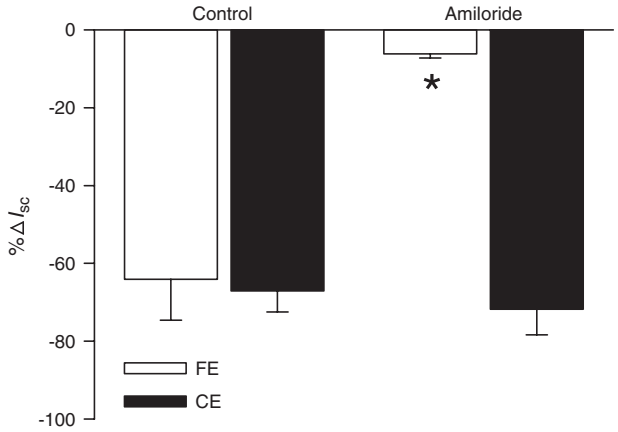

Fig. 5. Comparison of the effects of NPPB (3×10^{-5} M) on FE and CE in the absence and presence of amiloride. In the controls the responses to NPPB reflect the decrease from the basal level; in the presence of amiloride the results depict the decrease in I_{sc} from the level established by amiloride. $n=9$ and 8 separate experiments for FE and CE, respectively. *$P<0.05$.

micrographs (Fig. 1) prepared from CE with R_t of 1000 Ω or greater revealed a pseudo-stratified appearance with ciliated cells representing approximately 15–30% of the total population in most areas of the wells. In some areas of the wells ciliated cells were less prevalent. The appearance of CE did not mimic exactly FE morphology [see Fedan et al. (2000) for examples], in that CE was not as tall and fewer ciliated cells were present.

3.2. Basal bioelectric parameters for CE and FE

The basal spontaneous potential difference (SPD) values for CE and FE were not different (Table 1). However, basal I_{sc} values were significantly smaller in CE than in FE, while R_t values were significantly larger in CE.

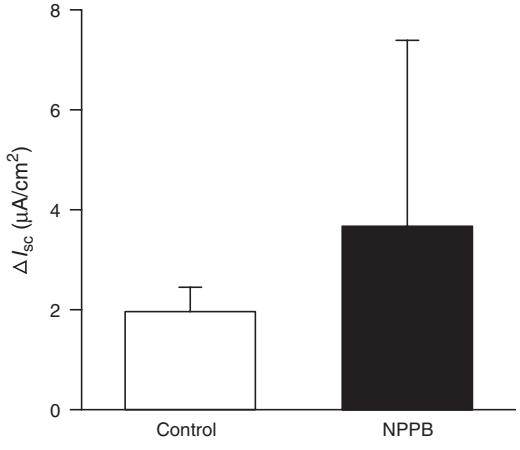

Fig. 6. Effect of apically-added NPPB (3×10^{-5} M) on responses of FE to basolaterally-administered MCh (3×10^{-7} M). Basal I_{sc} was 47.6±10.7 μA/cm^2 before the addition of MCh. NPPB caused a reduction in I_{sc} of 27.0±1.6 μA/cm^2. Responses in the absence (Control) and presence of NPPB were obtained in the same tracheal preparations. There was no effect of NPPB on the responses. $n=6$.

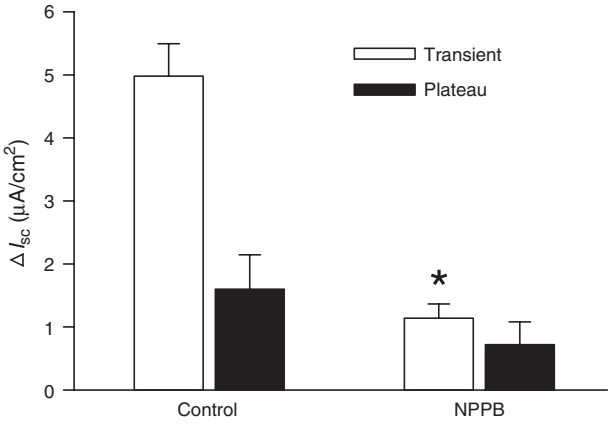

Fig. 7. Effect of apically-added NPPB (3×10^{-5} M) on responses of CE to basolaterally-administered MCh (3×10^{-7} M). Methacholine was administered after the response to NPPB became stable. Responses in the absence (Control) and presence of NPPB were obtained from separate culture dishes. Basal I_{sc} was 9.3±0.7 μA/cm^2 for the controls and 9.2±0.8 for the NPPB-treated cells. NPPB caused a reduction in I_{sc} of 6.1±0.6 μA/cm^2. *Significantly smaller than the phasic response obtained in the absence of NPPB. $n=20$ and 11 for the controls and NPPB-treated preparations, respectively.

3.3. Bioelectric responses to MCh

FE responded to basolaterally-applied MCh (3×10^{-7} M) with a monophasic increase in I_{sc} (Fig. 2); R_t was unaffected (before MCh, 102.5±9.7 Ω·cm^2; after MCh, 99.6±9.7 Ω·cm^2; $P=0.62$, $n=19$). CE responded to MCh with an initial transient increase in I_{sc} that was followed by a gradual increase to a sustained plateau (Fig. 2); R_t was unaffected (before MCh, 466.4±62.8 Ω·cm^2;

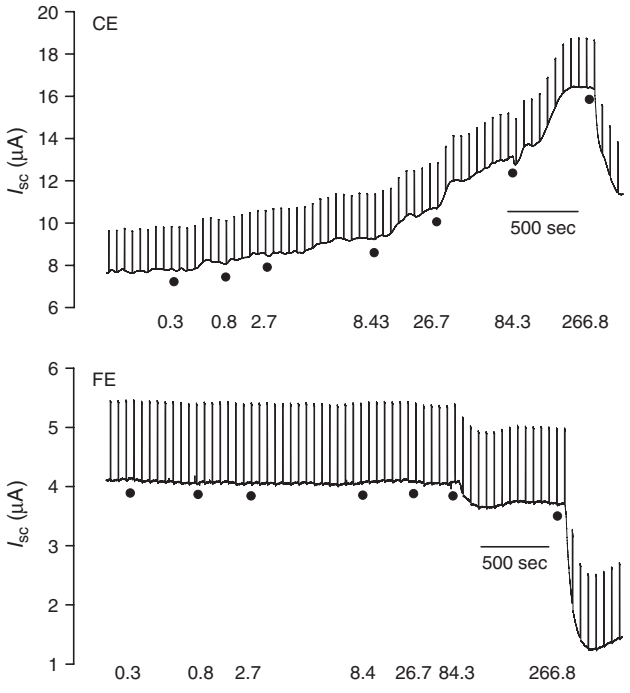

Fig. 8. Tracings showing the effects of cumulative apical additions of D-M on I_{sc} in CE (upper tracing) and FE (lower tracing) preparations. The numbers below each tracing are the D-M concentrations. These tracings are representative of $n=13$ (CE) and 11 (FE) experiments, which are summarized in Fig. 9.

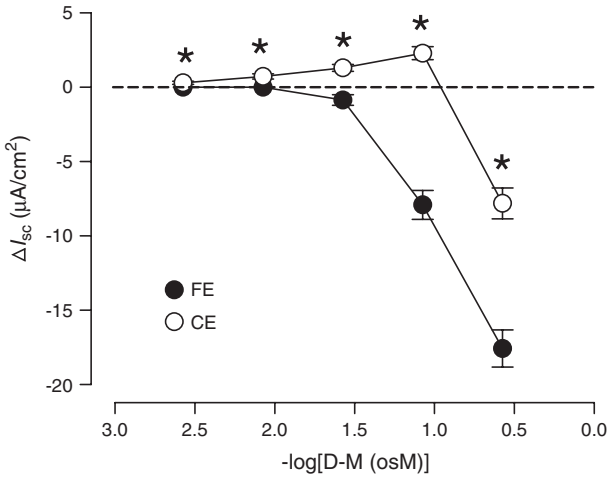

Fig. 9. Comparison of cumulative D-M concentration–response curves obtained from FE and CE preparations. Note the increase in I_{sc} in response of CE to the first four concentrations of D-M. *CE *vs.* FE, $P<0.05$.

after MCh, 455.0±57.5 Ω·cm^2; $P=0.53$, $n=8$). The results of several experiments are summarized in Fig. 3, where it can be seen that the transient responses of CE to MCh were significantly larger than the plateau responses of FE and CE.

3.4. Effects of ion transport blockers

As shown in Fig. 4, amiloride (apical, 3×10^{-5} M), a Na^+ channel blocker, evoked a rapid decrease in I_{sc} in FE (24.9± 3.9%; $n=9$) and CE (17.4±2.8%; $n=8$; FE *vs.* CE, $P=0.13$). R_t was unaffected by amiloride in FE and CE (Table 2). NPPB (apical, 3×10^{-5} M), a Cl^- channel blocker, reduced I_{sc} by *ca.* 65% in both FE and CE (Fig. 4). In contrast, NPPB in the presence of amiloride caused a modest decrease in I_{sc} in FE but a substantial decrease in I_{sc} in CE (Figs. 4 and 5). In the presence of amiloride, in FE NPPB increased R_t, but NPPB had no effect on R_t in CE (Table 2). Bumetanide (serosal, 10^{-5} M), a Na^+, K^+,2Cl^- co-transport inhibitor, decreased I_{sc} in both preparations (−16.6±4.9% and −24.4±5% in FE and CE, respectively; $P=0.29$; $n=6$), but did not affect R_t in either preparation (Table 2). Ouabain (serosal, 10^{-5} M), a Na^+,K^+-pump inhibitor, reduced I_{sc} in both FE and CE but did not affect R_t (Table 2). Lastly, iberiotoxin (serosal, 10^{-5} M), a Ca^+-activated, K^+-channel blocker, had no effect on I_{sc} or R_t in FE or CE (Table 2).

NPPB had no effect on responses of FE to basolateral MCh (Fig. 6). In CE, the transient response to MCh was inhibited significantly, whereas the plateau phase of the response was unaffected (Fig. 7).

3.5. Effects of hyperosmolar challenge

During cumulative additions of D-M to the apical chamber to elevate osmolarity, low and intermediate concentrations led to increases in I_{sc} in CE; however, I_{sc} was decreased by the highest concentration (Figs. 8 and 9). In intermediate concentrations of D-M in some CE preparations, transient decreases in I_{sc} occurred before stabilization of the response to a net increase (see response to 84.3 mosM in Fig. 8). These transients might reflect the effects of a greater concentration of D-M in the chamber before thorough mixing occurred in the reservoir and lines. R_t across CE was decreased by D-M (from 455±58 Ω·cm^2 in the absence of D-M to 238±25 Ω·cm^2 in the presence of 266.8 mosM D-M; $P<0.001$, $n=13$). When applied to FE, D-M caused only decreases in I_{sc} and did not affect R_t (109±11 Ω·cm^2 before addition of D-M and 88± 8 Ω·cm^2 in the presence of 266.8 mosM D-M; $P=0.133$, $n=11$). Transient decreases in I_{sc} were also seen in some FE preparations when intermediate concentrations of D-M were applied. With respect to its ability to decrease I_{sc}, D-M was approximately 3-fold less potent in CE compared to FE (Fig. 9).

4. Discussion

We compared the bioelectric properties of CE and FE with regard to basal parameters and responses to ion transport blockers, methacholine, and hyperosmolar challenge and observed that CE differed from FE in nearly every respect. CE were pseudostratified and well-differentiated; however, FE cells were more columnar and pseudostratified, and ciliated cells were more prevalent. Whereas the values of SPD did not differ, basal values for I_{sc} and R_t between the two preparations were different, with R_t making a greater contribution to SPD in CE than FE. MCh evoked monotonic increases in I_{sc} in FE, but complex responses in CE. NPPB's effects on I_{sc} were similar in FE and CE under basal conditions, but the blocker decreased I_{sc} to a greater extent in CE in the presence of amiloride. D-M evoked only decreases in I_{sc} in FE; in CE, D-M increased I_{sc} at low concentrations but decreased I_{sc} in the highest D-M concentration, and reduced R_t. In decreasing I_{sc}, D-M was *ca.* 3-fold more potent in FE than in CE.

The origin of the differences in the I_{sc} and R_t values in CE and FE is not apparent. Basal I_{sc} was reduced and R_t was increased in CE compared to FE. When compared to fresh airway epithelial cells from which cell cultures are prepared, the R_t values of cultured cells from many species and airway regions are typically larger than those measured in the originating fresh tissue (Van Scott, Cheng, Henke, & Yankaskas, 1991). It is conceivable that a decrease in the permeability of tight junctions occurred in CE, but we have no data to support this speculation. In our experiments, the reduced basal I_{sc} and greater effects of NPPB in CE in the presence of amiloride could suggest that electrogenic Na^+ transport was reduced and that a relative shift in Cl^- secretion had occurred. If this interpretation is correct, it is difficult to understand why NPPB was equi-effective in CE and FE in the absence of amiloride; future experiments are required to understand these amiloride–NPPB interactions. The increase in R_t in CE is not due to the contribution of the support matrix, as this resistance was removed electronically. In FE, resistance of epithelium-free preparations is negligible (unpublished findings). Thus, the electrical resistances of the epithelial substrata do not explain the differences in basal I_{sc} or R_t.

Several investigators have suggested that ion transport measured across airway epithelium is affected by the presence of submucosal glands. It is possible that the differences between FE and CE that we observed reflect the absence of the glands. Ballard, Fountain, Inglis, Corboz, and Taylor (1995) compared ion

transport in pig small bronchi and bronchioles with the prevalence of submucosal glands. These workers observed greater Cl^- transport in bronchi than in bronchioles. The prevalence of submucosal glands in the bronchi was seen to be greater in the bronchi than in the bronchioles, and the authors suggested that there may be a relationship between the magnitude of Cl^- transport and the presence of submucosal glands. Jarnigan, Davis, Bromberg, Gatzy, and Boucher (1983) found that rabbit tracheal epithelium, in which submucosal glands are scarce, is primarily Na^+-absorptive. These earlier findings could suggest that the differences between FE and CE could reflect the absence of submucosal glands in CE. In support of this interpretation is our finding that, in the absence of amiloride, NPPB had little effect on I_{sc}. However, arguing against this explanation was the finding that the transient response to MCh was inhibited by NPPB. Indeed, Jarnigan et al. (1983) found that acetylcholine did not evoke bioelectric responses in rabbit tracheal epithelium.

The I_{sc} responses of CE and FE to basolaterally-applied MCh could involve differences in its permeability through the two types of substrata. Stimulation of basolateral muscarinic receptors in CE would follow diffusion of the agonist only through the pores, which occupy a small fraction of the area of the matrix. The receptors of only a very small area of the basolateral membrane would be stimulated, at least initially. The diffusion barrier across the wall of FE includes the cartilage rings, the connective tissue between the rings, and the lamina propria. The lamina propria is not an appreciable diffusion barrier to MCh [Fedan and Frazer (1992) and unpublished findings]. It would be predicted that the onset of responses to MCh should have greater in FE than in CE. It is, therefore, unlikely that the lack of a transient response of FE to MCh can be explained by diffusion considerations.

Whereas the pre- and postjunctional localization and function of M_1-, M_2- and M_3-muscarinic receptor subtypes on nerves and smooth muscle of guinea-pig airways has been characterized (Eglen, Hegde, & Watson, 1996; Haddad, Landry, & Gies, 1991; Mak & Barnes, 1990; ten Berge, Roffel, & Zaagsma, 1995; Yang & Biggs, 1991; Zaagsma, Roffel, & Meurs, 1997), to our knowledge the functional characteristics of the subtypes in airway epithelium of guinea pigs and other species has not been studied. Mak and Barnes (1990) observed appreciable binding of [^{3}H](−) quinuclidinyl benzilate to tracheal smooth muscle in autoradiographic sections, but little binding to the epithelium. Clearly, functional muscarinic receptors exist in both FE and CE. In the perfused guinea-pig trachea preparation, the MCh concentration–response relationship for changes in V_t was biphasic, with lower concentrations evoking hyperpolarization and higher concentrations ($>$*ca.* 3×10^{-7} M) eliciting depolarization (Johnston et al., 2004). These two effects of MCh suggest the existence of multiple muscarinic receptors on the epithelial cells. Complex responses of the type seen here for CE have not been observed in FE at any concentration of MCh in Ussing chamber or isolated, perfused trachea preparations. It is not likely, therefore, that the perturbations required for mounting the trachea in the Ussing chambers results in the loss of characteristics responsible for CE-like behavior. Rather, the complex shape of responses by CE suggests that alterations in receptor type, prevalence, and/or transduction pathways had occurred. The selective inhibitory effect of NPPB on the phasic response of CE suggests an involvement of Cl^- channels in the phasic response.

Freed and Croxton (1993) compared the electrophysiological properties and responses to acetylcholine of canine tracheal epithelium that was adherent to the tracheal wall and contained smooth muscle with epithelium that had been separated from the airway wall. Basal R_t was greater (*ca*. 2-fold) in epithelium alone compared to the intact airway, whereas basal I_{sc} values did not differ. An increase in I_{sc} accompanied by a decrease in R_t in response to acetylcholine in epithelium alone was not evident in adherent epithelium, where acetylcholine did not induce an I_{sc} response but did increase R_t. These investigators concluded that the presence of airway smooth muscle or other components in the airway wall affects the electrophysiological properties of epithelium and its responses to acetylcholine. In the present study, R_t of CE was larger (*ca*. 4-fold) than that of FE, which is comparable to the results of Freed and Croxton. However, whereas there was no difference in I_{sc} in the absence and presence of airway wall in the former study, we observed that I_{sc} of unstimulated cells was *ca*. 4-fold larger in FE compared to CE, and that MCh had negligible effects on R_t in both preparations. It is important to note that smooth muscle was not present in FE. These comparisons indicate, first, that bioelectric responses of canine and guinea-pig epithelium to MCh, in the absence or presence of the airway wall, are different with respect to changes in I_{sc} and R_t, and second, that the differences between FE and CE are not attributable to the presence or absence of the airway wall.

Epithelial cells exhibit mechanisms for volume regulation (Lang et al., 1998), and hyperosmolar challenge induces shrinkage (Hjoberg, 1999; Willumsen, Davis, & Boucher, 1994; Wu et al., 2004). It could be reasonably expected that while some treatments might alter the cell volume set point, the primary transport and transductive pathways would be constitutive. Thus, it was surprising that the polarity of the bioelectric responses of CE to low concentrations of D-M was opposite that of FE. The end result was that D-M is less potent in CE than in FE. D-M may be potentially capable of stimulating two pathways in epithelial cells, one which results in an increase in I_{sc} and one which results in a decrease. Is the former one up-regulated in CE? Is it related to cell volume regulation and/or EpDRF release? Does the interplay between two opposing processes stimulated by D-M determine its potency as an initiator of bioelectric responses? Additional experiments are required to address these questions.

In conclusion, CE are not a surrogate for FE. Studies performed solely on cultured airway epithelial cells, without validation of their functional identity to fresh cells, might give rise to results that do not mimic the *in vivo* features of this tissue.

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