BJP British Journal of Pharmacology

DOI:10.1111/bph.12027
www.brjpharmacol.org

RESEARCH PAPER

Benzimidazolones enhance the function of epithelial Na^+ transport

M Laube[1], SU Kimpel[2], P Dietl[3], UH Thome[1] and OH Wittekindt[3]

[1]*Division of Neonatology, University of Leipzig, Leipzig, Germany,* [2]*Department of Pediatrics, University of Ulm, Ulm, Germany, and* [3]*Department of General Physiology, University of Ulm, Ulm, Germany*

Correspondence
Ulrich Thome, Division of Neonatology, University of Leipzig, Liebigstraße 20a, 04103 Leipzig, Germany. E-mail: uhthome@web.de

Keywords
ussing chamber; short-circuit current; epithelial sodium transport; patch clamp; E-BIO

Received
27 September 2011
Revised
11 September 2012
Accepted
18 September 2012

BACKGROUND AND PURPOSE

Pharmacological enhancement of vectorial Na^+ transport may be useful to increase alveolar fluid clearance. Herein, we investigated the influence of the benzimidazolones 1-ethyl-1,3-dihydro-2-benzimidazolone (1-EBIO), 5,6-dichloro-1-EBIO (DC-EBIO) and chlorzoxazone on vectorial epithelial Na^+ transport.

EXPERIMENTAL APPROACH

Effects on vectorial Na^+ transport and amiloride-sensitive apical membrane Na^+ permeability were determined by measuring short-circuit currents (I_{SC}) in rat fetal distal lung epithelial (FDLE) monolayers. Furthermore, amiloride-sensitive membrane conductance and the open probability of epithelial Na^+ channels (ENaC) were determined by patch clamp experiments using A549 cells.

KEY RESULTS

I_{SC} was increased by approximately 50% after addition of 1-EBIO, DC-EBIO and chlorzoxazone. With permeabilized basolateral membranes in the presence of a 145:5 apical to basolateral Na^+ gradient, the benzimidazolones markedly increased amiloride-sensitive I_{SC}. 5-(N-Ethyl-N-isopropyl)amiloride-induced inhibition of I_{SC} was not affected. The benzamil-sensitive I_{SC} was increased in benzimidazolone-stimulated monolayers. Pretreating the apical membrane with amiloride, which inhibits ENaC, completely prevented the stimulating effects of benzimidazolones on I_{SC}. Furthermore, 1-EBIO (1 mM) and DC-EBIO (0.1 mM) significantly increased (threefold) the open probability of ENaC without influencing current amplitude. Whole cell measurements showed that DC-EBIO (0.1 mM) induced an amiloride-sensitive increase in membrane conductance.

CONCLUSION AND IMPLICATIONS

Benzimidazolones have a stimulating effect on vectorial Na^+ transport. The antagonist sensitivity of this effect suggests the benzimidazolones elicit this action by activating the highly selective ENaC currents. Thus, the results demonstrate a possible new strategy for directly enhancing epithelial Na^+ transport.

Abbreviations

$amil_{max}$, maximal amiloride-sensitive I_{SC}; CFTR, cystic fibrosis transmembrane conductance regulator; ENaC, epithelial Na^+ channel; FDLE, fetal distal lung epithelia; I_{amil}, amiloride-sensitive current; I_{ouab}, ouabain-sensitive current; I_{SC}, short-circuit current; NP_O, product of the number of active channels (N) and open probability (P_O) in a patch; RDS, respiratory distress syndrome; R_{te}, trans-epithelial resistance

Introduction

Alveolar cells constantly transport Na^+ ions from the apical to the basolateral side, creating an osmotic gradient for the movement of fluid out of the alveolar space (Matalon and O'Brodovich, 1999). Na^+ enters the cells through epithelial Na^+ channels (ENaC) in the apical membrane and is extruded by Na,K-ATPases in the basolateral membrane. This active

vectorial Na^+ transport is mainly responsible for alveolar fluid clearance and crucial for preventing pulmonary oedema (Hummler *et al.*, 1996; Ware and Matthay, 2001). Furthermore, survival of adults with the acute respiratory distress syndrome (ARDS) is related to the activity of their alveolar fluid clearance (Ware and Matthay, 2001). Preterm infants with respiratory distress syndrome (RDS) have reduced airway epithelial Na^+ transport (Barker *et al.*, 1997) and reduced ENaC expression (Helve *et al.*, 2004) compared with preterm infants without RDS or term infants, and decreased alveolar fluid clearance has been found to contribute to the pathogenesis of RDS (O'Brodovich, 1996). Therefore, mechanisms or treatments to up-regulate lung epithelial vectorial Na^+ transport and alveolar fluid clearance may decrease the morbidity of preterm infants and adults with RDS. However, aside from β-receptor agonists (Perkins *et al.*, 2006), no pharmacological treatment to enhance vectorial epithelial Na^+ transport is available so far.

Potassium channels located in the basolateral membrane determine the cell's resting potential (Wilson *et al.*, 2006) and recycle the K^+ entering the cells by the Na,K-ATPase. Hence, K^+ channels control the driving force for Na^+ entry through the apical membrane into the cytosol and for Cl^- exit into the apical space. Blockade of K^+ channels has been shown to diminish Na^+ absorption (Leroy *et al.*, 2004) as well as Cl^- secretion (Devor *et al.*, 1997). In addition, substances known to activate K^+ channels of the SK and IK type, such as the benzimidazolones 1-EBIO, DC-EBIO and chlorzoxazone, were shown to increase Cl^- secretion in cells expressing wild-type cystic fibrosis transmembrane conductance regulator (CFTR) (Singh *et al.*, 2000; 2001) and were discussed as possible therapeutic targets for cystic fibrosis. However, in human CFT1 airway cells, which are homozygous for ΔF508-CFTR, vectorial Na^+ transport was stimulated instead, thus worsening the Na^+ hyperabsorption known to occur in CFTR-deficient epithelia (Gao *et al.*, 2001).

Substances capable of increasing vectorial Na^+ transport (Wilson *et al.*, 2006) may become useful for treating RDS (O'Brodovich, 1996; Matalon *et al.*, 2002). We initially sought to determine whether activating K^+ channels would indeed increase amiloride-sensitive short-circuit current and thus vectorial Na^+ transport in monolayers of rat fetal distal lung epithelia (FDLE). The benzimidazolones 1-EBIO, DC-EBIO and chlorzoxazone are known to be activators of Ca^{2+}-gated potassium channels of the SK and IK type (Syme *et al.*, 2000). Unexpectedly, our results indicate that in addition to their K^+-channel activating properties, the benzimidazolones tested also stimulated amiloride-sensitive epithelial Na^+ transport and thus highlight a possible new pathway to pharmacologically stimulate alveolar fluid clearance.

Methods

Tissue preparation

All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All animal care and experimental procedures were approved by the Institutional Review Boards (Regierungspräsidium Tübingen and Landesdirektion Leipzig). Sprague–Dawley rats were bred at Charles River Laboratories (Kisslegg, Germany) and the medical experimental centre (MEZ) of the University of Leipzig (Leipzig, Germany). The animals were housed in rooms with a controlled temperature (22°C), humidity (55%) and 12 h light–dark cycle. Food and water were available *ad libitum*. The pregnant rats were killed by carbon dioxide inhalation.

FDLE cells were isolated from lungs of 18–20 days gestation rat foetuses as described previously (Jassal *et al.*, 1991; Thome *et al.*, 2001; 2003). In brief, minced lungs were digested in a solution containing 0.125% trypsin (Life Technologies, Darmstadt, Germany) and 0.4 $mg \cdot mL^{-1}$ DNAse (CellSystems, Troisdorf, Germany) in Eagle's minimum essential medium (MEM, Biochrom, Berlin, Germany) for 10 min. Digestion was stopped by the addition of MEM containing 10% FBS (PAA Laboratories, Cölbe, Germany). Cells were collected by centrifugation (440× *g*) and resuspended in 15 mL MEM containing 0.1% collagenase (CellSystems) and DNAse. This solution was incubated for 15 min at 37°C. The collagenase activity was neutralized by the addition of 15 mL MEM containing 10% FBS. The cells were plated twice for 1.5 h to remove contaminating fibroblasts. The supernatant contained epithelial cells with >95% purity (Jassal *et al.*, 1991). Cells were cultured in MEM with 10% FBS, glutamine (2 mM, PAA Laboratories), penicillin (100 $U \cdot mL^{-1}$; Life Technologies), streptomycin (100 $\mu g \cdot mL^{-1}$, Life Technologies) and amphotericin B (0.25 $\mu g \cdot mL^{-1}$; Life Technologies).

A549 cells, a human alveolar carcinoma cell line purchased from the American Type Culture Collection (ATCC) at passage number 81, were kept in DMEM-F12 (Biochrom) with glutamine (2 mM) and 10% FBS and passaged twice weekly.

Measurement of bioelectric properties of FDLE monolayers

For Ussing chamber experiments, FDLE cells were seeded on Costar Snapwell no. 3407 (Corning Inc., Corning, NY) at a density of 10^6 cells per insert. Experiments were performed on the fourth or fifth day of culture. The Ussing chambers were filled with a solution containing (in mM) Na^+ 145, K^+ 5, Ca^{2+} 1.2, Mg^{2+} 1.2, Cl^- 125, HCO_3^- 25, $H_2PO_4^-$ 3.3, HPO_4^{2-} 0.8 (pH 7.4). Furthermore, the basolateral side contained 10 mM glucose. On the apical side, 10 mM mannitol was used instead, to minimize the possible contribution of a putative apical Na^+-glucose co-transporter to Na^+ influx (Icard and Saumon, 1999). The ionic composition was identical on both sides unless stated otherwise. The solutions were continuously bubbled with a mixture of 95% O_2 and 5% CO_2 and warmed to 37°C. I_{SC} (short-circuit current) was measured every 20 s with a trans-epithelial voltage clamp (Physiologic Instruments, San Diego, CA). Square wave pulses (±2 mV, 500 ms) were applied across the monolayers every 20 s, allowing calculation of trans-epithelial resistance (R_{te}) from the current change using Ohm's law. Amiloride-sensitive current (ΔI_{SC}) was determined as the decrease in I_{SC} after addition of 10 μM amiloride to the apical compartment of the Ussing chamber and used as a measure of ENaC activity. Ouabain was added at a concentration of 1 mM to the basolateral compartment to determine the I_{SC} decrease after blocking of the Na,K-ATPases. Concentration–response relationships were recorded by cumulative addition of the agonist.

To measure the inhibitory effects of benzamil and 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) on the current 10 μM benzamil or 100 μM EIPA were added to the apical compartment, and the antagonist-sensitive I_{SC} was determined. All antagonist-sensitive currents were measured after the current had reached a plateau. As a measure of CFTR activity, the $CFTR_{inh}172$-sensitive I_{SC} (10 μM) was determined accordingly.

To determine apical Na^+ permeability independent of changes in Na,K-ATPase activity and basolateral K^+ conductance, 140 mM of basolateral Na^+ was replaced by 116 mM *N*-methyl-D-glucamine and 24 mM choline, both assumed to be impermeant cations, resulting in a 145:5 apical-to-basolateral Na^+ gradient. After the basal I_{SC} had been recorded, the basolateral membrane was permeabilized by adding 100 μM amphotericin B (a pore-forming antibiotic) to the basolateral side of the Ussing chamber, thus incapacitating active transport and permitting equilibration of intracellular Na^+ with the solution in the basolateral compartment. Following this treatment, all of the I_{SC} is due to passive Na^+ flux through apical Na^+-conductive pathways down the Na^+ concentration gradient from the apical to the basolateral side (Guo *et al.*, 1998; Thome *et al.*, 2001; 2003). As soon as the increase in I_{SC} induced by addition of amphotericin B had reached a maximum, its amiloride-sensitive component ($amil_{max}$) was determined by adding 10 μM amiloride to the apical compartment. In additional experiments, an inverse gradient was used resulting in a 5:145 apical-to-basolateral Na^+ gradient. The basolateral membrane was permeabilized by adding 100 μM amphotericin B and amiloride applied afterwards. Thereby, we sought to ensure that currents were not biased by an incomplete permeabilization of the basolateral membrane.

Experiments with benzimidazolones and control experiments were always performed simultaneously and on the same batches of cells and had the same duration and timeframes. Multiple wells were derived from each cell isolation procedure. Ussing chamber experiments were included in the analysis only when the R_{te} exceeded 200 Ω°Cm^2 throughout the experiment.

Patch clamp studies

Patch clamp studies were performed on A549 cells of passages 85–95 that were seeded on glass coverslips. On the second or third day post seeding, the cells were mounted in a bath on the stage of an inverted microscope (Zeiss Axiovert 135, Zeiss, Oberkochen, Germany) and perfused with a solution containing (mM): Na^+ 135, K^+ 5, Ca^{2+} 1, Mg^{2+} 4, Cl^- 10, gluconate 135, HEPES 10, glucose 5, mannitol 10 (pH 7.4). Cell attached and whole cell currents were measured with an EPC9 patch clamp amplifier (Heka Elektronik, Lambrecht, Germany). A standard personal computer running Pulse software (Heka) controlled the EPC9 and stored the current tracings.

Cell attached recordings

Patch pipettes were pulled from quartz capillaries with 1 mm outer diameter and 0.7 mm inner diameter (Sutter, Novato, CA) using a P2000 laser puller (Sutter) and filled with a solution containing (mM): Na^+ 135, Mg^{2+} 5, Cl^- 10, gluconate 135, HEPES 10, glucose 5 (ph 7.4), resulting in a tip resistance between 5 and 10 MΩ. After forming a gigaseal, cells were depolarized by switching the bath perfusion to a solution containing (mM) K^+ 135, Mg^{2+} 5, Cl^- 10, gluconate 135, HEPES 10, glucose 5, mannitol 20 (pH 7.4). Currents were recorded in cell attached mode at –100 mV membrane potential and room temperature, filtered at 2 kHz and sampled at 5 kHz.

Whole cell recordings

Patch pipettes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK) with 1.5 mm outer diameter using a Zeitz micropipette puller (Zeitz Instruments, Martinsried, Germany), fire-polished and filled with a solution containing (mM): Na^+ 130, Ca^{2+} 0.506, Mg^{2+} 2.741, Cl^- 6.494, EGTA 10, gluconate 130, HEPES 10, glucose 5 (pH 7.4), resulting in a tip resistance between 3 and 5 MΩ. After a gigaseal had been formed, intracellular access was established by suction. Whole cell currents were recorded at membrane potentials between –100 and +60 mV in 20 mV increments, filtered at 2.5 kHz and sampled at 10 kHz. Recordings were analysed with Pulsefit software (Heka).

Data analysis and statistical procedures

Significant differences among groups treated with the different substances and controls were determined by ANOVA and Dunnett's *post hoc* test or by Mann–Whitney *U*-test.

To determine concentration–response relationships of benzimidazolone-stimulated I_{SC}, the resulting currents of each benzimidazolone concentration step were fitted to the following equation using GraphPad Prism (GraphPad Software, La Jolla, CA):

$$y = E_{min} + [(E_{max} - E_{min})/(1 + 10\,\hat{}\,((\mathrm{LogEC}_{50} - x) \times n_H))],$$

with

y = effect
E_{max} = maximal achieved current
E_{min} = lowest current
EC_{50} = concentration eliciting half of the maximal effect
x = log(concentration)
n_H = Hill coefficient (slope of curve)

Cell attached recordings containing typical ENaC openings (Lazrak *et al.*, 2000a) were analysed with QUB software (Research Foundation, State University, Buffalo, NY). Traces were digitally filtered at 200 Hz and idealized using the Segmental-K-Means algorithm (Qin, 2004) using a minimal dwell time of 3 ms. From amplitude histograms, open probability (P_O), mean open and mean closed times were calculated. The product of the number of active channels (N) and the P_o in a patch (NP_o), representing the activity of channels was calculated from cell attached recordings as follows:

$$NP_o = \sum_{i=0}^{N} i \times \frac{t_i}{T}$$

where T is the total recording time, i is the number of open channels and t_i is the recording time during which i channels were open (Lazrak *et al.*, 2000b). Amplitude, NP_o and mean open/closed times before and after addition of benzimidazolones were compared using the Mann–Whitney *U*-test.

Materials

Amiloride (A-7410, Sigma, Chemical Company, St. Louis, MO), amphotericin B (A-4888, Sigma), benzamil (B-2417,

Sigma), $CFTR_{inh}172$ (3430, Tocris, Bristol, UK), chlorzoxazone (C-4397, Sigma), DC-EBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2-benzimidazolone) (1422, Tocris), 1-EBIO (1-ethyl-1,3-dihydro-2-benzimidazolone) (SML0034, Sigma), EIPA (5-(*N*-Ethyl-*N*-isopropyl)amiloride) (A-3085, Sigma), *N*-methyl-D-glucamine ($NMDG^+$, M-2004, Sigma), ouabain (O-3125, Sigma). Amiloride, ouabain and NMDG were dissolved in water. All other drugs were either prepared in 100% ethanol or DMSO. The presence of these solvents did not have a significant effect on the responses evoked.

Results

Electrophysiological studies of complete monolayers

All FDLE monolayers used in the electrophysiological studies were obtained from 14 different cell isolations (comprising the foetuses of 42 pregnant rats). The mean R_{te} was 846 ± 20 Ω·cm² (mean ± SEM). Of these, 95% maintained an R_{te} > 200 Ω·cm² throughout the experiment and were included in the analysis.

I_{SC} (μA cm^{-2}) was significantly increased by 1-EBIO (3 mM, ANOVA, $P < 0.05$), by DC-EBIO (0.3 mM, ANOVA, $P < 0.001$) and by chlorzoxazone (1 mM, ANOVA, $P < 0.001$), while I_{SC} in control monolayers remained stable at baseline value (Figure 1A). Likewise, the amiloride-sensitive I_{SC} and ouabain-sensitive I_{SC} were increased by 1-EBIO (ANOVA, $P < 0.01$), DC-EBIO and chlorzoxazone (Figure 1B). Ouabain-sensitive I_{SC} was only slightly higher than amiloride-sensitive I_{SC}. Basolaterally applied ouabain in the presence of amiloride reduced the remaining amiloride-insensitive I_{SC} only slightly. Taken together, the analysis showed that amiloride-sensitive and ouabain-sensitive I_{SC}s were considerably higher in monolayers stimulated with benzimidazolones than in control monolayers.

The augmentation of I_{SC} elicited by stepwise increasing concentrations of 1-EBIO and DC-EBIO resulted in the concentration–response curves shown in Figure 2. Thus, DC-EBIO was more potent than 1-EBIO by about 1.5 orders of magnitude. The Hill coefficient is larger than 1 and therefore implies some form of binding cooperativity for 1-EBIO and DC-EBIO. The coefficients fitted are listed in Table 1.

Since different amiloride-sensitive Na^+ channels might be present in FDLE cells, the antagonists EIPA and benzamil were employed to determine the effect of benzimidazolones on highly selective ENaCs. EIPA is known to block non-selective Na^+ channels, whereas benzamil inhibits highly selective Na^+ channels. The EIPA-sensitive I_{SC} did not differ between benzimidazolone-stimulated and control monolayers (Figure 3). However, the benzamil-sensitive I_{SC} was significantly increased in benzimidazolone-stimulated monolayers (*t*-test, $P < 0.05$).

To determine the apical Na^+ permeability independent of the Na, K-ATPase, the basolateral membrane was permeabilized in the presence of an apical to basolateral 145:5 Na^+ gradient. The basolateral application of amphotericin B induced, as expected, a sharp increase in I_{SC}, which was mostly amiloride-sensitive (Figure 4). Only a small current remained after addition of amiloride. The increase in I_{SC} induced by amphotericin B (Figure 5A) and the maximal amiloride-sensitive I_{SC} ($amil_{max}$) (Figure 5B) were significantly higher in monolayers that had been treated with benzimidazolones before permeabilization compared with control

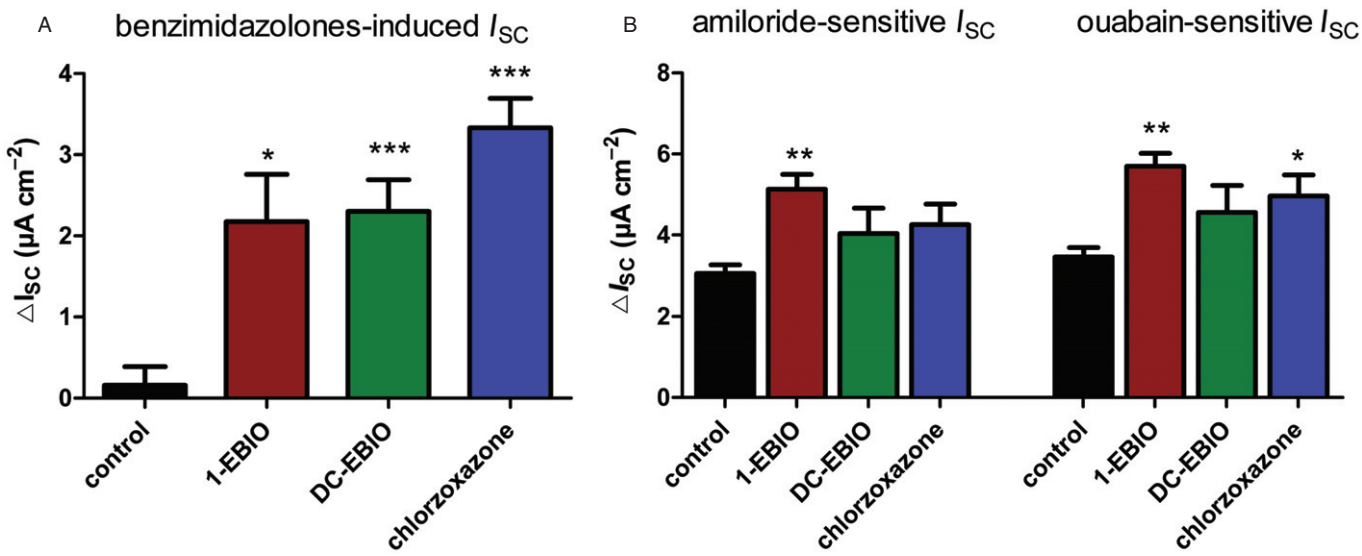

Figure 1

Benzimidazolones increase short-circuit currents. Effects of 1-EBIO (3 mM), DC-EBIO (0.3 mM) and chlorzoxazone (1 mM) on I_{SC} of rat FDLE cell monolayers. The figure shows the increase in I_{SC} induced by addition of one of the compounds tested (benzimidazolone-induced I_{SC}, A), the I_{SC} decrease after adding amiloride (10 μM, the amiloride-sensitive I_{SC} (B) and the I_{SC} decrease after addition of amiloride and ouabain (1 mM, ouabain-sensitive I_{SC} (B). Mean treatment-associated changes in current ± SEM of 22 (control), 6 (1-EBIO), 16 (DC-EBIO) and 16 (chlorzoxazone) monolayers are shown and were significantly increased by the substances tested (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by Dunnett's *post hoc* test vs. control).

Table 1

Fitting results of concentration–response relationships

	pEC_{50}	E_{min} (μA/cm²)	E_{max} (μA/cm²)	n_H
1-EBIO	3.27 ± 0.49	4.34 ± 0.88	6.23 ± 1.04	1.91
DC-EBIO	4.51 ± 0.24	4.41 ± 0.41	6.09 ± 0.53	2.30

pEC_{50} = negative decadic logarithm of the EC_{50} value (concentration eliciting half of the maximal effect), E_{min} = lowest current, E_{max} = maximal achieved current, n_H = Hill coefficient (slope of curve) Similar EC_{50} values were reported for the stimulation of Cl^- secretion (Singh *et al.*, 2001; Hamilton and Kiessling, 2006) and for activation of I_K and S_K type potassium channels (Jensen *et al.*, 1998; Hougaard *et al.*, 2007; John *et al.*, 2007). Mean ± SEM.

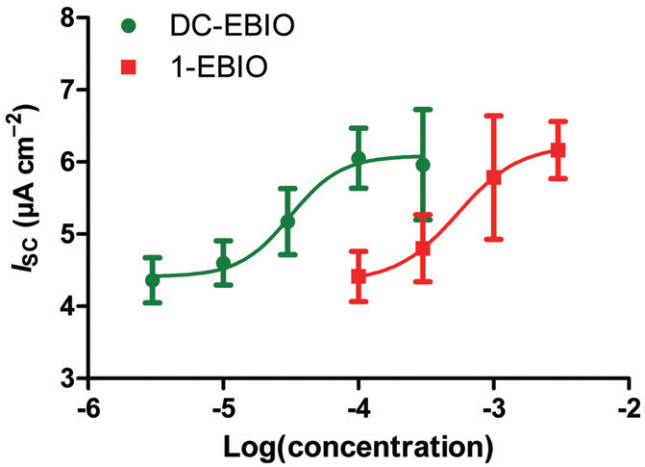

Figure 2

DC-EBIO is equally effective as and more potent than 1-EBIO. 1-EBIO and DC-EBIO were applied cumulatively at increasing concentrations to determine concentration–response relationships for the action on FDLE monolayers. The *x*-axis shows the decadic logarithm of concentrations, the *y*-axis the resulting I_{SC}. The solid lines represent the best fit of a modified Hill function to the data sets with parameters of the fits presented in Table 1. Data represent 6 monolayers for 1-EBIO and 12 monolayers for DC-EBIO. Error bars represent SEM.

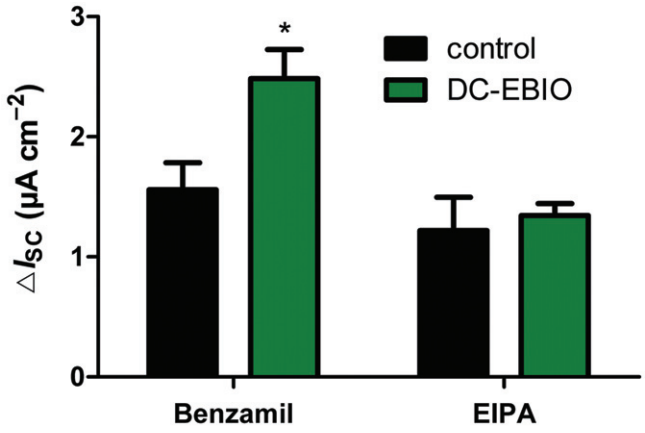

Figure 3

Benzimidazolones enhance benzamil-sensitive I_{SC}. Effects of DC-EBIO (0.3 mM) on benzamil- and EIPA-sensitive I_{SC} of rat FDLE cell monolayers. The figure shows the current reduction caused by 10 μM benzamil and 100 μM EIPA. Mean treatment-associated changes in current ± SEM of 12 (control/benzamil), 9 (DC-EBIO/benzamil), 11 (control/EIPA) and 14 (DC-EBIO/EIPA) monolayers (*$P < 0.05$ by *t*-test vs. control).

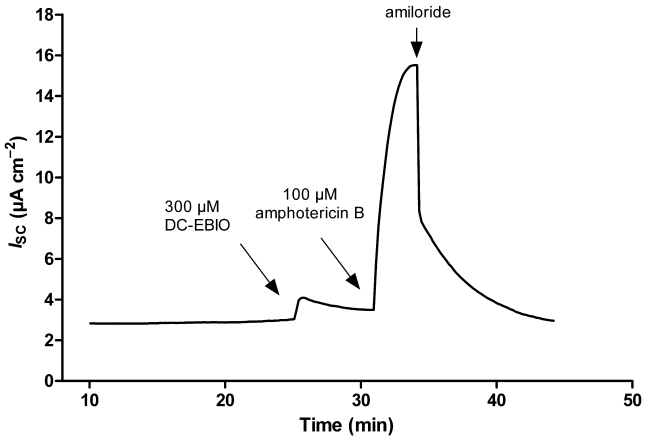

Figure 4

I_{SC} trace of the permeabilized basolateral membrane measurement. After addition of DC-EBIO (300 μM) amphotericin B (100 μM) was applied basolaterally. At the maximum current increase, amiloride (10 μM) was added apically to determine max. amiloride-sensitive I_{SC}.

experiments without benzimidazolones ($amil_{max}$: 1-EBIO, ANOVA, $P < 0.01$; DC-EBIO, ANOVA, $P < 0.001$; chlorzoxazone, ANOVA, $P < 0.001$). In addition, experiments with an inverse Na^+ gradient also showed a threefold increase in $amil_{max}$ in monolayers treated with benzimidazolones before permeabilization (data not shown).

To determine the involvement of an increase in ENaC activity in the stimulating effects of benzimidazolones, the apical membrane was pre-blocked with amiloride before the addition of DC-EBIO (0.3 mM). The inhibition of ENaC by amiloride reduced the I_{SC}, as expected, which was subsequently not altered by application of DC-EBIO. Therefore, inhibition of ENaC completely prevented the DC-EBIO-induced increase in current (Figure 6).

The effect of benzimidazolones on CFTR activity was analysed in FDLE cells. The I_{SC} was inhibited by addition of amiloride to the apical compartment and then stimulated with DC-EBIO (0.3 mM). The selective inhibitor of CFTR channels, $CFTR_{inh}172$, was applied to the apical compartment

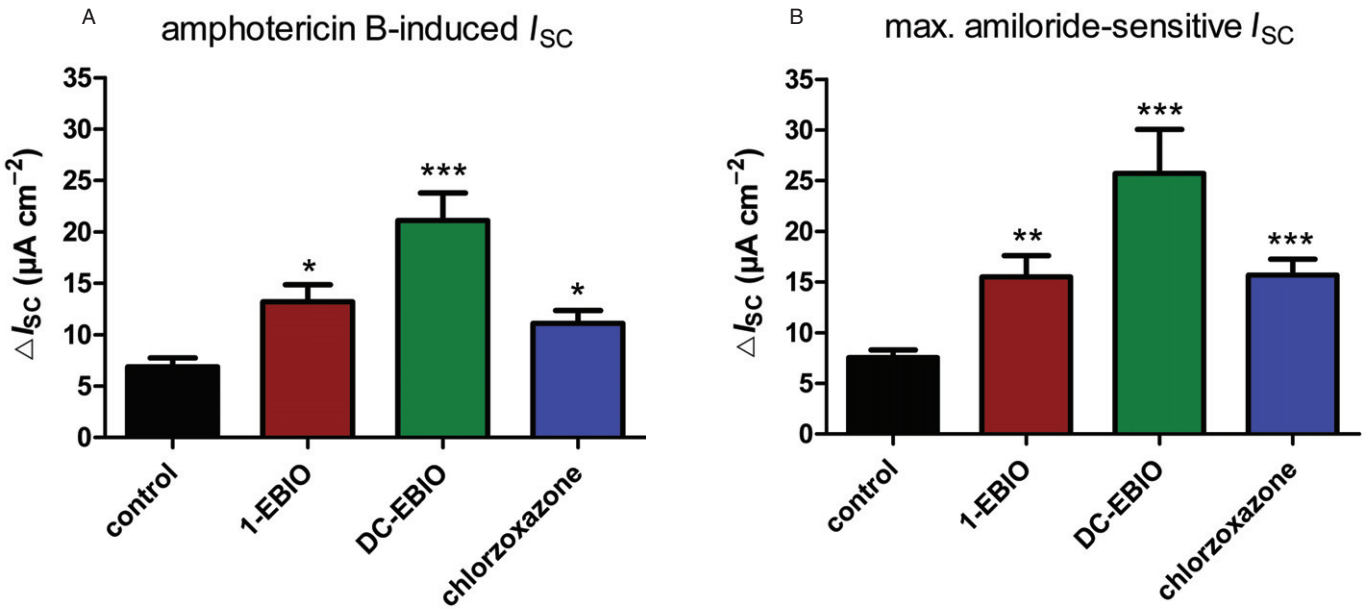

Figure 5

Benzimidazolones enhance the magnitude of the amiloride-sensitive apical conductance. The monolayers were subjected to a 145:5 apical to basolateral Na^+ gradient, the basolateral membrane was permeabilized by addition of 100 µM amphotericin B, resulting in an amphotericin B-induced increase in I_{SC} (A). The maximum amiloride-sensitive I_{SC} ($amil_{max}$, B) reduction was caused by the addition of 10 µM amiloride into the apical compartment at the maximum current increase. The data of 13 (control), 5 (1-EBIO), 3 (DC-EBIO) and 12 (chlorzoxazone) monolayers are represented as mean ± SEM (**P < 0.01; ***P < 0.001 by Dunnett's *post hoc* test vs. control).

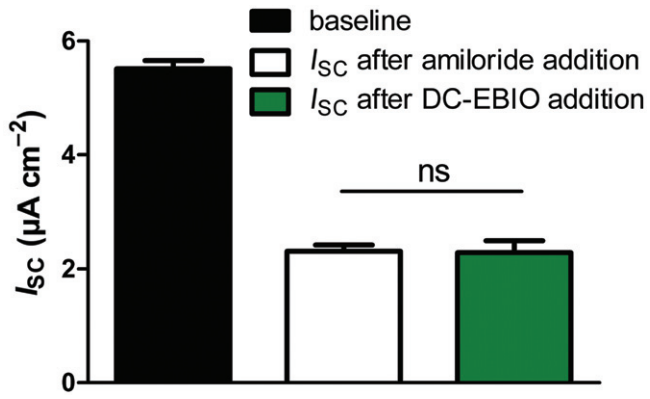

Figure 6

DC-EBIO given after amiloride does not affect I_{SC}. The columns show the I_{SC} after mounting the monolayers in the Ussing chambers (baseline), I_{SC} after addition of amiloride and I_{SC} after adding DC-EBIO (0.3 mM). Mean currents ± SEM of 14 monolayers are shown. After blocking the ENaC with amiloride, DC-EBIO did not significantly alter the I_{SC} (ns).

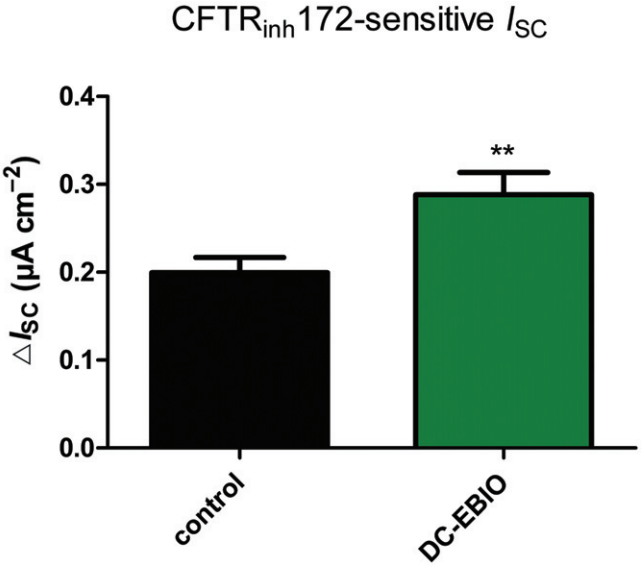

Figure 7

DC-EBIO enhances the magnitude of $CFTR_{inh}172$-sensitive currents. The figure shows the $CFTR_{inh}172$-sensitive I_{SC} after amiloride inhibition and DC-EBIO (0.3 mM) stimulation compared with control monolayers without DC-EBIO stimulation. Mean $CFTR_{inh}172$-sensitive currents ± SEM of 23 (control) and 20 (DC-EBIO) monolayers are shown; the $CFTR_{inh}172$-sensitive I_{SC} was much smaller than the amiloride-sensitive I_{SC} but significantly increased by DC-EBIO treatment (**P < 0.01 by t-test vs. control).

and the $CFTR_{inh}172$-sensitive I_{SC} between the DC-EBIO-stimulated monolayers and controls was determined. The DC-EBIO stimulated monolayers showed an increased $CFTR_{inh}172$-sensitive I_{SC} compared with control monolayers (t-test, P < 0.01) (Figure 7). However, the difference in $CFTR_{inh}172$-sensitive I_{SC} between DC-EBIO stimulated and

Table 2

Mean closed and mean open times of cell attached recordings from A549 cells analysed with QUB software

Mean closed time		Mean open time	
Control	**1-EBIO**	**Control**	**1-EBIO**
49.2 ± 11.7 s	19.2 ± 3.8 s	0.81 ± 0.28 s	0.86 ± 0.14 s
Control	**DC-EBIO**	**Control**	**DC-EBIO**
56.3 ± 19.9 s	26.1 ± 12.5 s	0.53 ± 0.24 s	0.55 ± 0.11 s

Membrane patches were recorded under control conditions followed by the addition of 1-EBIO (1 mM) or DC-EBIO (100 μM). Mean ± SEM.

control monolayers was less than 0.1 μA cm^{-2}, showing that the proportion of CFTR-activity in I_{SC} of FDLE cells was rather small.

Electrophysiological studies of single cells

Cell attached recordings from A549 cells at a membrane potential of –100 mV revealed inwardly conducting channels with a current amplitude of 0.4 pA. Assuming a reversal potential close to zero the slope conductance of this current amplitude corresponds to a conductance of 4 pS, which is typical for the highly selective cation channel. In these patches, NP_o was tripled, while current amplitude remained unchanged after addition of 1-EBIO (1 mM) or DC-EBIO (100 μM) to the bathing solution (Figure 8A). The NP_o was significantly increased by 1-EBIO ($P < 0.05$) whereas DC-EBIO just showed the same tendency (Figure 8B). Several other patches that did not reveal any channel openings longer than 3 ms before benimidazolone application revealed considerable channel activity thereafter. However, these patches were not included in the statistical analysis. Some other patches were quiescent and remained so in the presence of 1-EBIO and DC-EBIO. One patch showed an 80 pS channel, which was not further investigated. The mean closed times decreased after addition of 1-EBIO or DC-EBIO, whereas mean open times were not significantly altered after stimulation with 1-EBIO or DC-EBIO. These values are presented in Table 2. The decrease in mean closed times was statistically significant for 1-EBIO compared with control (Mann–Whitney *U*-test, $P < 0.05$) (Figure 8C).

Whole cell patch clamp recordings of A549 cells resulted in almost linear *I*/*V* relationships. Reversal potentials were around –5 mV owing to the almost symmetrical solutions (high Na^+ in patch pipette and bath). Whole cell membrane conductance was markedly increased by 10 and 100 μM DC-EBIO, an effect completely abolished by 10 μM amiloride (Figure 9).

Discussion and conclusions

Pharmacological activation of transepithelial Na^+ reabsorption is expected to be beneficial in the treatment of severe lung failure with impaired alveolar fluid clearance. However, pharmacologically useful substances to activate transepithelial Na^+ reabsorption are not yet available, except the rather non-specific and second-messenger-dependent β-receptor agonists and proteases. This study demonstrates the pharmacological activation of transepithelial Na^+ reabsorption in alveolar cells by benzimidazolone derivatives.

The benzimidazolone derivatives used in this study were previously known as activators of Ca^{2+}-dependent K^+ channels of the SK and IK type, but not B_K (Devor *et al.*, 1996a; Jensen *et al.*, 1998; Singh *et al.*, 2000; Syme *et al.*, 2000; Pedarzani *et al.*, 2001). As such, they hyperpolarize the cell membrane. The substances differ mainly in their EC_{50} values, not in their electrophysiological actions (Syme *et al.*, 2000; Cao *et al.*, 2001; Singh *et al.*, 2001). In the case of chlorzoxazone, its K^+ channel activating properties are used clinically for decreasing muscle tone (Elenbaas, 1980). In epithelial cells, membrane potential is also controlled by Ca^{2+}-dependent K^+ channels (Ridge *et al.*, 1997) and determines the driving force for passive influx of Na^+ and exit of Cl^- (O'Brodovich and Rafii, 1993; Devor *et al.*, 1996a; 1997; MacVinish *et al.*, 1998; Sakuma *et al.*, 1998; Singh *et al.*, 2000; Leroy *et al.*, 2004). Since Na^+ entry appears to be the rate-limiting step (Benos *et al.*, 1992; Stutts *et al.*, 1995), an increased driving force for Na^+ entry may ultimately lead to increased vectorial Na^+ transport, as shown in human cystic fibrosis CFT1 airway cells homozygous for ΔF508-CFTR (Gao *et al.*, 2001) as well as in a study testing the effects of Na,K-ATPase overexpression (Thome *et al.*, 2001).

However, in the experiments described here, several lines of evidence indicate that a more direct action on epithelial sodium channels appears to play the primary role in eliciting the observed increased transepithelial Na^+ transport in distal lung epithelial cells.

In patch clamp studies, the benzimidazolones induced a threefold increase in the open probability as well as a decrease in mean closed times of cation channels, which resembled the functional properties of the epithelial Na^+ channel. Furthermore, the whole cell measurements suggest the increased current induced by the benzimidazolones is sodium-selective and concentration-dependent and it was inhibited by an amiloride concentration commonly used to block ENaC almost selectively. Experiments with permeabilized basolateral membrane in the presence of a transepithelial ionic gradient clearly demonstrated that the amiloride-sensitive apical membrane conductance is strongly increased by the benzimidazolones tested. These latter experiments allowed

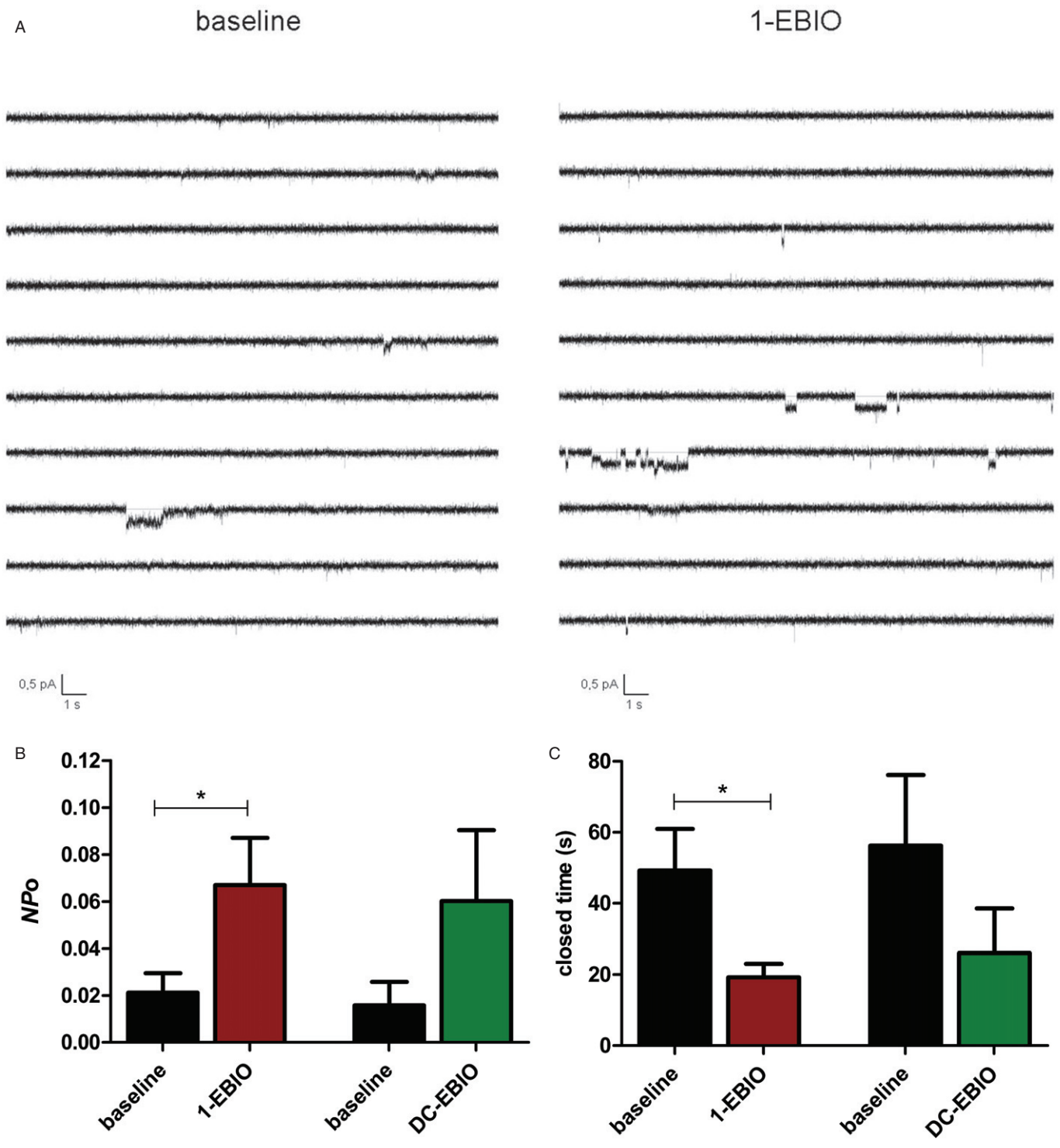

Figure 8

Benzimidazolones increase the open probability in cell attached recordings. (A) Typical cell attached recording traces of an A549 cell at –100 mV membrane potential before (left) and after addition of 1-EBIO (right) to the bathing solution. (B) Summary of data of NP_O before and after adding 1-EBIO ($n = 9$) or DC-EBIO ($n = 5$) to the bathing solutions. The increase in NP_O was statistically significant for 1-EBIO (*$P < 0.05$). (C) Mean closed time before and after addition of 1-EBIO ($n = 9$) or DC-EBIO ($n = 5$) (*$P < 0.05$). Data represented as mean ± SEM.

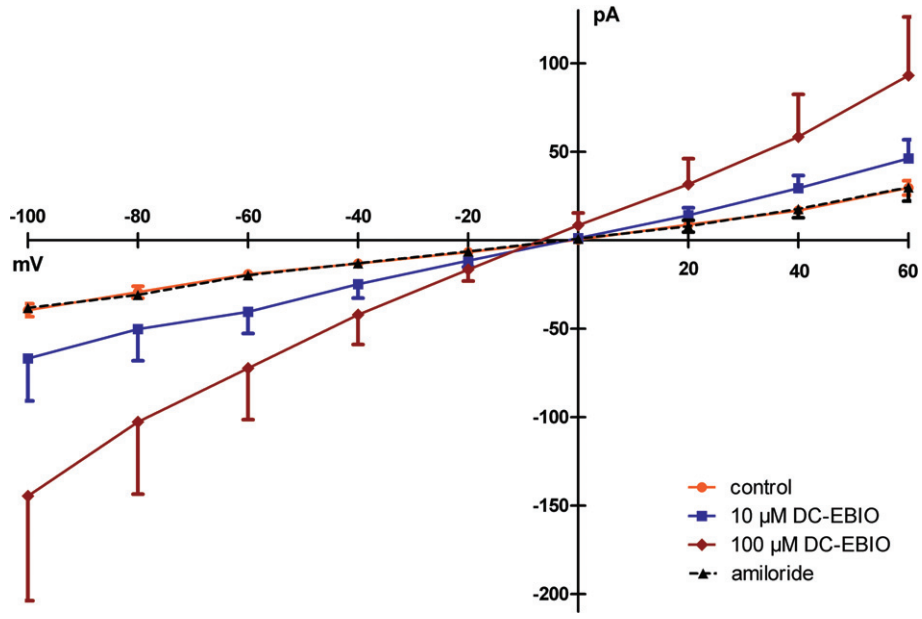

Figure 9

DC-EBIO enhances membrane conductance in whole cell measurements. Whole cell current measurements in A549 cells ($n = 7$) with Na^+ as the only permeant ion on either side of the cell membrane. Addition of 10 and 100 µM DC-EBIO resulted in a concentration-dependent increase in cell membrane conductance, which was almost completely amiloride-sensitive. Currents were normalized by the membrane conductance under control conditions and shown as mean ± SEM.

the assessment of apical membrane properties without the usual interference of basolateral electrogenic activity, basolaterally located K^+ channels or intracellular–extracellular K^+ gradients (Devor *et al.*, 1996b). These results suggest that there is a connection between benzimidazolones exposure and ENaC activity. In accord with this, further short-circuit measurements corroborated the amiloride sensitivity of benzimidazolones-stimulated currents. The amiloride-insensitive currents were similar regardless of the previously elicited effects of benzimidazolones in the same monolayers. In addition, the experiment shown in Figure 6 demonstrated that benimidazolones had no effect when added in the presence of amiloride. Benzamil-sensitive currents were increased by the benzimidazolones tested while EIPA-sensitive currents were not altered by the benzimidazolone derivates, indicating the benzimidazolones enhanced highly selective Na^+ channels.

We also found that stimulation with benzimidazolone induced a small, but not significant, increase in the Tram34-sensitive $I_{SC,}$ which supposedly represents a small increase in IK channel activity and leads to membrane hyperpolarization and thus increased Na^+ transport (data not shown), as has been described in CFT1 cells. The magnitude of this effect, however, appeared to be very small in our wild-type cell model. We did not detect any effect of the benzimidazolones on scyllatoxin-sensitive I_{SC} or UCL1684-sensitive I_{SC} (both blockers of SK channels, data not shown).

Increases in I_{SC} following benzimidazolone administration were also described in CFT1 cells and were attributed to increased K^+ channel conductance because no change in apical membrane conductance was observed (Gao *et al.*, 2001). This is in contrast to the results described here. However, CFT1 cells were derived from the trachea of a CF patient with homozygous ΔF508-CFTR and thus lacked the regulatory influence of CFTR on ENaC, resulting in constitutively high ENaC activity (Stutts *et al.*, 1995). Wild-type alveolar cells, on the other hand, have normal and functional CFTR and thus a much lower baseline ENaC activity. We speculate that the high ENaC activity in CFT1 cells is already at a maximum level and thus cannot be further increased, unlike the regulated activity in the wild-type cells studied here, which would sufficiently explain the discrepant results. The same interpretation was used in another study showing that ENaC activity of *CFTR* (–/–) mice could not be further increased by forskolin or trypsin supposedly because ENaC was already maximally activated (Lazrak *et al.*, 2011). Since the binding site of benzimidazolones in any channel they activate is still unknown, it is possible that these substances act by interfering with the same site that CFTR uses to regulate ENaC. CFTR itself is also modulated by benzamidazolones, as shown by the analysis of CFTR-activity using $CFTR_{inh}172$ in FDLE cells. Whether this modulation was a direct action on CFTR or mediated through hyperpolarization, as a result of activation of K^+ channels, was not determined.

In our Na^+ transport measurements, however, the contribution of Cl^- conducting channels such as CFTR to the effects of the benzimidazolones was negligible for three reasons: firstly, the $CFTR_{inh}172$-sensitive component of I_{SC} in FDLE cells was very small; secondly, in our patch clamp studies, almost no chloride was present; and thirdly, ouabain, which blocks all active transport, did not reduce the I_{SC} considerably more than amiloride alone, thus indicating that the amiloride-insensitive Cl^- secretion was negligible. Interfer-

ence from K^+ channels was small as shown by the small effect of K channel blockers and further limited in patch clamp studies by replacing K^+ by Na^+, which is also the reason why the reversal potential was close to 0.

Since the short-circuit and whole cell currents elicited by the benzimidazolone derivatives were highly sensitive to amiloride and benzamil, but not EIPA, and cell attached currents showed a current amplitude and gating behaviour typical of the 4 pS version of ENaC (Benos *et al.*, 1996; Lazrak *et al.*, 2000a; Jain *et al.*, 2001; Lazrak and Matalon, 2003), it is supposed that the observed effect of the benzimidazolone derivatives is induced by activation of ENaC channels. However, other amiloride-sensitive cation conductances exist in certain cells (Ramminger *et al.*, 2004) and may play a part.

The mechanisms of action of benzimidazolones on any channels are currently unknown, as well as the binding sites. In this study, the cell attached recordings suggest that benzimidazolones penetrated the cell membrane and acted from the inside of the cell, as the outside was inaccessible after tight seal formation. In small conductance Ca^{2+} activated K channels, benzimidazolones act by shifting the Ca^{2+} concentration–response curve to the left (Pedarzani *et al.*, 2001).

The stimulating effect of benzimidazolones on transepithelial Na^+ reabsorption may be explained by direct activation of existing Na^+ conducting channels, by activation of silent channels or by the insertion of extra channels into the cell membrane. However, the activating effects of the benzimidazolone derivatives were observed in whole cell patch clamp experiments as well as in cell-attached recordings, in which the benzimidazolone derivatives tested increased the NP_o. Furthermore, the onset of action was rather fast, which makes it unlikely that the insertion of new channels contributes to this effect. Therefore, increased activation of channels already present in the apical membrane, resulting in an increased open probability, is the most likely explanation. The activated channels may include previously silent channels or channels with low activity at baseline. Similar effects were previously shown for the actions of the compounds on the IK and SK channels (Devor *et al.*, 1996a; Singh *et al.*, 2000; Syme *et al.*, 2000). Furthermore, the EC_{50} values obtained in this study are within the range reported for the stimulation of Cl^- secretion (Singh *et al.*, 2001; Hamilton and Kiessling, 2006) and for the activation of I_K- and S_K- type potassium channels (Jensen *et al.*, 1998; Hougaard *et al.*, 2007; John *et al.*, 2007).

None of the substances tested here is likely to be useful for improving lung function clinically. Relatively high concentrations were needed to produce an effect, which are likely to induce unacceptable side effects owing to their K^+ channel activating properties, which include depression of nerve cell excitability and smooth and skeletal muscle relaxation causing a drop in blood pressure. However, this study clearly shows that transepithelial Na^+ reabsorption can be activated by suitable molecules. Human ENaC activation has recently been described following application of capsazepin (Yamamura *et al.*, 2004) and a novel synthetic compound (Lu *et al.*, 2008). Further research may clarify the binding sites and mechanisms of action of benzimidazolones and be used to develop more selective molecules for activation of transepithelial Na^+ transport, as has been demonstrated for CFTR (Caci *et al.*, 2003).

Acknowledgements

The authors wish to thank Tatiana Felder, Uwe Kirchner and Melanie Timmler for excellent technical assistance and George Chandy for encouraging us in this project.

Supported by: DFG Th626 (to UHT), D1402 (to PD), 6th framework of the EU, Pulmo-Net (to PD) and Medizinische Fakultät, University of Ulm, P770 (to OHW).

Conflicts of interest

None.

References

Barker PM, Gowen CW, Lawson EE, Knowles MR (1997). Decreased sodium ion absorption across nasal epithelium of very premature infants with respiratory distress syndrome. J Pediatr 130: 373–377.

Benos DJ, Cunningham S, Baker RR, Beason KB, Oh Y, Smith PR (1992). Molecular characteristics of amiloride-sensitive sodium channels. Rev Physiol Biochem Pharmacol 120: 31–113.

Benos DJ, Awayda MS, Berdiev BK, Bradford AL, Fuller CM, Senyk O *et al.* (1996). Diversity and regulation of amiloride-sensitive Na+ channels. Kidney Int 49: 1632–1637.

Caci E, Folli C, Zegarra-Moran O, Ma T, Springsteel MF, Sammelson RE *et al.* (2003). CFTR activation in human bronchial epithelial cells by novel benzoflavone and benzimidazolone compounds. Am J Physiol Lung Cell Mol Physiol 285: L180–L188.

Cao Y, Dreixler JC, Roizen JD, Roberts MT, Houamed KM (2001). Modulation of recombinant small-conductance Ca(2+)-activated K(+) channels by the muscle relaxant chlorzoxazone and structurally related compounds. J Pharmacol Exp Ther 296: 683–689.

Devor DC, Singh AK, Frizzell RA, Bridges RJ (1996a). Modulation of Cl- secretion by benzimidazolones. I. Direct activation of a Ca(2+)-dependent K+ channel. Am J Physiol 271 (5 Pt 1): L775–L784.

Devor DC, Singh AK, Bridges RJ, Frizzell RA (1996b). Modulation of Cl- secretion by benzimidazolones. II. Coordinate regulation of apical GCl and basolateral GK. Am J Physiol 271 (5 Pt 1): L785–L795.

Devor DC, Singh AK, Gerlach AC, Frizzell RA, Bridges RJ (1997). Inhibition of intestinal Cl- secretion by clotrimazole: direct effect on basolateral membrane K+ channels. Am J Physiol 273 (2 Pt 1): C531–C540.

Elenbaas JK (1980). Centrally acting oral skeletal muscle relaxants. Am J Hosp Pharm 37: 1313–1323.

Gao L, Yankaskas JR, Fuller CM, Sorscher EJ, Matalon S, Forman HJ *et al.* (2001). Chlorzoxazone or 1-EBIO increases Na(+) absorption across cystic fibrosis airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 281: L1123–L1129.

Guo Y, DuVall MD, Crow JP, Matalon S (1998). Nitric oxide inhibits Na+ absorption across cultured alveolar type II monolayers. Am J Physiol 274 (3 Pt 1): L369–L377.

Hamilton KL, Kiessling M (2006). DCEBIO stimulates Cl- secretion in the mouse jejunum. Am J Physiol Cell Physiol 290: C152–C164.

Helve O, Pitkanen OM, Andersson S, O'Brodovich H, Kirjavainen T, Otulakowski G (2004). Low expression of human epithelial sodium channel in airway epithelium of preterm infants with respiratory distress. Pediatrics 113: 1267–1272.

Hougaard C, Eriksen BL, Jorgensen S, Johansen TH, Dyhring T, Madsen LS *et al.* (2007). Selective positive modulation of the SK3 and SK2 subtypes of small conductance Ca2+-activated K+ channels. Br J Pharmacol 151: 655–665.

Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A *et al.* (1996). Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. Nat Genet 12: 325–328.

Icard P, Saumon G (1999). Alveolar sodium and liquid transport in mice. Am J Physiol 277 (6 Pt 1): L1232–L1238.

Jain L, Chen XJ, Ramosevac S, Brown LA, Eaton DC (2001). Expression of highly selective sodium channels in alveolar type II cells is determined by culture conditions. Am J Physiol Lung Cell Mol Physiol 280: L646–L658.

Jassal D, Han RN, Caniggia I, Post M, Tanswell AK (1991). Growth of distal fetal rat lung epithelial cells in a defined serum-free medium. In Vitro Cell Dev Biol 27A: 625–632.

Jensen BS, Strobaek D, Christophersen P, Jorgensen TD, Hansen C, Silahtaroglu A *et al.* (1998). Characterization of the cloned human intermediate-conductance Ca2+-activated K+ channel. Am J Physiol 275 (3 Pt 1): C848–C856.

John VH, Dale TJ, Hollands EC, Chen MX, Partington L, Downie DL *et al.* (2007). Novel 384-well population patch clamp electrophysiology assays for Ca2+-activated K+ channels. J Biomol Screen 12: 50–60.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Lazrak A, Matalon S (2003). cAMP-induced changes of apical membrane potentials of confluent H441 monolayers. Am J Physiol Lung Cell Mol Physiol 285: L443–L450.

Lazrak A, Samanta A, Venetsanou K, Barbry P, Matalon S (2000a). Modification of biophysical properties of lung epithelial Na(+) channels by dexamethasone. Am J Physiol Cell Physiol 279: C762–C770.

Lazrak A, Samanta A, Matalon S (2000b). Biophysical properties and molecular characterization of amiloride-sensitive sodium channels in A549 cells. Am J Physiol Lung Cell Mol Physiol 278: L848–L857.

Lazrak A, Jurkuvenaite A, Chen L, Keeling KM, Collawn JF, Bedwell DM *et al.* (2011). Enhancement of alveolar epithelial sodium channel activity with decreased cystic fibrosis transmembrane conductance regulator expression in mouse lung. Am J Physiol Lung Cell Mol Physiol 301: L557–L567.

Leroy C, Dagenais A, Berthiaume Y, Brochiero E (2004). Molecular identity and function in transepithelial transport of K(ATP) channels in alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol 286: L1027–L1037.

Lu M, Echeverri F, Kalabat D, Laita B, Dahan DS, Smith RD *et al.* (2008). Small molecule activator of the human epithelial sodium channel. J Biol Chem 283: 11981–11994.

MacVinish LJ, Hickman ME, Mufti DA, Durrington HJ, Cuthbert AW (1998). Importance of basolateral K+ conductance in maintaining Cl- secretion in murine nasal and colonic epithelia. J Physiol 510 (Pt 1): 237–247.

Matalon S, O'Brodovich H (1999). Sodium channels in alveolar epithelial cells: molecular characterization, biophysical properties, and physiological significance. Annu Rev Physiol 61: 627–661.

Matalon S, Lazrak A, Jain L, Eaton DC (2002). Invited review: biophysical properties of sodium channels in lung alveolar epithelial cells. J Appl Physiol 93: 1852–1859.

McGrath J, Drummond G, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

O'Brodovich H, Rafii B (1993). Effect of K channel blockers on basal and beta-agonist stimulated ion transport by fetal distal lung epithelium. Can J Physiol Pharmacol 71: 54–57.

O'Brodovich HM (1996). Immature epithelial Na+ channel expression is one of the pathogenetic mechanisms leading to human neonatal respiratory distress syndrome. Proc Assoc Am Physicians 108: 345–355.

Pedarzani P, Mosbacher J, Rivard A, Cingolani LA, Oliver D, Stocker M *et al.* (2001). Control of electrical activity in central neurons by modulating the gating of small conductance Ca2+-activated K+ channels. J Biol Chem 276: 9762–9769.

Perkins GD, McAuley DF, Thickett DR, Gao F (2006). The beta-agonist lung injury trial (BALTI): a randomized placebo-controlled clinical trial. Am J Respir Crit Care Med 173: 281–287.

Qin F (2004). Restoration of single-channel currents using the segmental k-means method based on hidden Markov modeling. Biophys J 86: 1488–1501.

Ramminger SJ, Richard K, Inglis SK, Land SC, Olver RE, Wilson SM (2004). A regulated apical Na(+) conductance in dexamethasone-treated H441 airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 287: L411–L419.

Ridge FP, Duszyk M, French AS (1997). A large conductance, Ca2+-activated K+ channel in a human lung epithelial cell line (A549). Biochim Biophys Acta 1327: 249–258.

Sakuma T, Takahashi K, Ohya N, Nakada T, Matthay MA (1998). Effects of ATP-sensitive potassium channel opener on potassium transport and alveolar fluid clearance in the resected human lung. Pharmacol Toxicol 83: 16–22.

Singh AK, Devor DC, Gerlach AC, Gondor M, Pilewski JM, Bridges RJ (2000). Stimulation of Cl(-) secretion by chlorzoxazone. J Pharmacol Exp Ther 292: 778–787.

Singh S, Syme CA, Singh AK, Devor DC, Bridges RJ (2001). Benzimidazolone activators of chloride secretion: potential therapeutics for cystic fibrosis and chronic obstructive pulmonary disease. J Pharmacol Exp Ther 296: 600–611.

Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC *et al.* (1995). CFTR as a cAMP-dependent regulator of sodium channels. Science 269: 847–850.

Syme CA, Gerlach AC, Singh AK, Devor DC (2000). Pharmacological activation of cloned intermediate- and small-conductance Ca(2+)-activated K(+) channels. Am J Physiol Cell Physiol 278: C570–C581.

Thome U, Chen L, Factor P, Dumasius V, Freeman B, Sznajder JI *et al.* (2001). Na,K-ATPase gene transfer mitigates an oxidant-induced decrease of active sodium transport in rat fetal ATII cells. Am J Respir Cell Mol Biol 24: 245–252.

Thome UH, Davis IC, Nguyen SV, Shelton BJ, Matalon S (2003). Modulation of sodium transport in fetal alveolar epithelial cells by oxygen and corticosterone. Am J Physiol Lung Cell Mol Physiol 284: L376–L385.

Ware LB, Matthay MA (2001). Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med 163: 1376–1383.

Wilson SM, Brown SG, McTavish N, McNeill RP, Husband EM, Inglis SK *et al*. (2006). Expression of intermediate-conductance, Ca2+-activated K+ channel (KCNN4) in H441 human distal airway epithelial cells. Am J Physiol Lung Cell Mol Physiol 291: L957–L965.

Yamamura H, Ugawa S, Ueda T, Nagao M, Shimada S (2004). Capsazepine is a novel activator of the delta subunit of the human epithelial Na+ channel. J Biol Chem 279: 44483–44489.