Expression and function of the epithelial sodium channel δ -subunit in human respiratory epithelial cells *in vitro*

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ABSTRACT

Using human airway epithelial cell lines (i.e. NCI-H441 and Calu-3) as well as human alveolar epithelial type I-like (ATI) cells in primary culture, we studied the contribution of the epithelial sodium channel δ -subunit (δ -ENaC) to transepithelial sodium transport in human lung *in vitro*. Endogenous δ -ENaC protein was present in all three cell types tested, however, protein abundance was low and no expression was detected in the apical cell membrane of these cells. Similarly, known modulators of δ -ENaC activity, such as capsazepine and icilin (activators) and Evans blue (inhibitor) did not show effects on short-circuit current (I_{sc}), suggesting that δ -ENaC is not involved in the modulation of transcellular sodium absorption in NCI-H441 cell monolayers. Over-expression of δ -ENaC in NCI-H441 cells resulted in detectable protein expression in the apical cell membrane, as well as capsazepine and icilin-stimulated increases in I_{sc} that were effectively blocked by Evans blue, and that were consistent with δ -ENaC activation and inhibition, respectively. Consequently, these observations suggest that δ -ENaC expression is low in NCI-H441, Calu-3, and ATI cells and does not contribute to transepithelial sodium absorption.

Keywords: Airway epithelium; Alveolar epithelium; ENaC; Ussing chamber; Sodium absorption

INTRODUCTION

The epithelial surface of the lungs is lined with the airway surface liquid (ASL). In the upper airways, an optimal height of ASL is a prerequisite for the mucociliary clearance. Concerted apical chloride secretion and sodium absorption, mainly mediated by the cystic fibrosis conductance regulator (CFTR) and epithelial sodium channels (ENaC) determine transepithelial water flow across the airway epithelial barrier and, consequently the ASL height ($\sim 8 \mu\text{m}$) [42]. In the alveoli, maintaining an extremely thin ($\sim 0.1 \mu\text{m}$) layer of lining fluid is necessary to allow effective gas exchange [5]. Alveolar fluid clearance (AFC) is mainly driven by active sodium absorption via ENaC channels across the distal lung epithelium. Impaired AFC is associated with pathological conditions, i.e. the formation of pulmonary oedema [14].

Four ENaC subunits, which share 27-37% homology on amino acid level, have been described in mammals: the pore-forming α - and δ - as well as the accessory β - and γ -ENaC. The most obvious difference between these classic α -, β -, γ -ENaC subunits and δ -ENaC is their distinct tissue distribution. Alpha-, β -, and γ -ENaC are abundantly expressed in sodium-absorbing epithelia of kidney, colon and lung and a number of other epithelia [8, 10, 11, 16, 29]. In contrast, the highest transcriptional expression levels of δ -ENaC were detected in non-epithelial tissues, such as brain, pancreas, heart, testis and ovary, whilst lung, kidney, colon and several other tissues showed only low expression [43, 45]. A new transcript, which was named ENaC δ_2 , was recently found to be expressed in several human tissues, including the CNS [20]. Additionally, the pharmacological and biophysical properties are clearly distinct between δ - and α -ENaC-generated currents. For example, δ -ENaC is about 30-times less sensitive to amiloride and its analogue, benzamil ($K_{0.5}(\text{amiloride}) = 2.6 \mu\text{M}$ and $0.08 \mu\text{M}$, $K_{0.5}(\text{benzamil}) = 270$ and 7 nM for δ - and α -ENaC, respectively) but more selective for sodium *versus* lithium, whereas α -ENaC is more selective for lithium *versus* sodium [43, 28].

The physiological importance of α -, β - and γ -ENaC subunits in the lung has long been established. Alpha-ENaC is essential for AFC at birth, as neonatal α -ENaC knockout mice died from the inability to clear their lungs of fluid [26]. In adult rats, silencing of α -ENaC using siRNA reduced the basal AFC levels and abrogated the stimulation of AFC by β -agonists [33]. The roles of β - and γ -ENaC subunits in the alveoli appear less critical. Mice lacking β - or γ -ENaC were able to clear their lungs at birth but died from abnormal renal electrolyte re-absorption [4, 19, 36].

Delta-ENaC has been found expressed in several human respiratory epithelial cell lines, i.e. A549, NCI-H441 and 16HBE14o- as well as in ATII cells in primary culture and in human lung tissue [27, 37, 50]. Moreover, Bangel-Ruland *et al.* [3] demonstrated the presence of δ -ENaC in primary human nasal epithelial cells (HNE). Since δ -ENaC physically interacts with α - and γ -subunits in NCI-H441 cells, and $\delta\alpha\beta\gamma$ -channels, heterologously expressed in *X. laevis* oocytes, displayed biophysical and pharmacological characteristics (i.e. ion selectivity, single channel sodium conductance, affinity to amiloride, gating kinetics) distinct from those of $\alpha\beta\gamma$ - and $\delta\beta\gamma$ -channels, it has been suggested that δ -ENaC may contribute to the variety of amiloride-sensitive currents across the native alveolar epithelia by forming different combinations of heteromeric channels with classic ENaC subunits [27, 28]. Because the molecular basis for the distinct sodium conductances detected in native respiratory epithelial cells has not been identified, the possible contribution of δ -ENaC-generated currents to the net sodium transport is of a particular interest. However, the fact that δ -ENaC in rodents is a pseudogene hinders the development of experimental animal models, thus *in vitro* approaches are currently the most promising to elucidate the physiological function of the ion channel [21].

In this study, we aimed to test the hypothesis that δ -ENaC contributes to transepithelial ion transport, and to determine its physiological role in the human airway epithelium *in vitro*. To this end, we utilised common cell models of human lung epithelium, i.e. NCI-H441, a human

lung adenocarcinoma cell line in which δ -ENaC expression has been described previously at both mRNA and protein levels [27, 37], Calu-3, a human bronchial adenocarcinoma cell line, and human ATI-like cells in primary culture. To identify δ -ENaC-mediated currents, selective modulators of δ -ENaC function that have been characterised previously in *X. laevis* oocytes [46-48] were used. Whilst amiloride and benzamil only allow distinguishing between the channel complexes on the basis of the higher affinities to the α -subunit [43], the δ -ENaC inhibitor, Evans blue ($IC_{50} = 143 \mu M$), and the activators, capsazepine and icilin ($EC_{50} = 8 \mu M$ and $33 \mu M$, respectively) modulate δ -ENaC channel activity with only marginally affecting α -ENaC-mediated currents [46-48]. Here, we describe the δ -ENaC expression in human airway and alveolar epithelial cells and the paradoxical absence of δ -ENaC-specific activity in these cells *in vitro*.

MATERIALS AND METHODS

Materials

We used and compared two primary anti- δ -ENaC antibodies: a commercially available antibody raised against amino acids 81-310 near the N-terminus of human δ -ENaC (SC-21015, Santa Cruz Biotechnology, Heidelberg, Germany, termed anti- δ -N in this report) and an antibody raised against the C-terminus of the δ -ENaC protein (amino acids 563-638; this sequence is not conserved in other ENaC subunits, but common to both described δ -ENaC isoforms [20], termed anti- δ -C in this report). For immunofluorescence microscopy, matching Alexa Fluor-488-labelled F(ab')₂ fragments were supplied by Invitrogen (Biosciences, Dun Laoghaire, Ireland). All cell culture plastics were obtained from Greiner BioOne (Frickenhausen, Germany), with the exception of Transwell Clear inserts (12 mm in diameter, pore size 0.4 μ m; Corning, VWR, Dublin, Ireland). All cell culture media and reagents were from Sigma-Aldrich (Dublin, Ireland), apart from Gibco RPMI 1640 medium (Invitrogen) and Small Airway Growth Medium (SAGM, Lonza, Verviers, Belgium). All other chemicals and reagents were of the highest purity grade available and purchased from Sigma-Aldrich unless stated otherwise.

Cell culture

All cell types were cultured in a humidified atmosphere at 37°C in 5% CO₂. The medium was changed every other day. The transepithelial electrical resistance (TEER) of cell monolayers grown under liquid-covered conditions on Transwell Clear inserts was measured using a Millicell ERS Volt-Ohm meter equipped with STX-2 electrodes (Millipore, Carrigtwohill, Ireland), and corrected for the background value contributed by the Transwell Clear inserts and medium.

Human bronchial epithelial Calu-3 cells (ATCC HTB-55) were obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Cells were seeded at a density

of 100,000/cm² on Transwell Clear inserts and grown in Eagle's minimum essential medium (EMEM), supplemented with 10% FBS, 100 U/ml penicillin, 100 µM/ml streptomycin, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 15 mM glucose. Ussing chamber studies were carried out on days 14-21 in culture, all other experiments on day 12.

The human bronchiolar epithelial NCI-H441 cell line (ATCC HTB-174) was purchased from LGC Standards, Teddington, UK. NCI-H441 cells were seeded at a density of 250,000/cm² on Transwell Clear inserts and cultured in Gibco RPMI 1640 medium, supplemented with 10% FBS, 100 U/ml penicillin, 100 µM/ml streptomycin and 1 mM sodium pyruvate. Twenty-four hours post-seeding, the medium was replaced with RPMI medium additionally supplemented with 200 nM dexamethasone and ITS supplement (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, Roche Diagnostics Limited, West Sussex, UK) [39]. Experiments with NCI-H441 cells were carried out on days 8-14 in culture.

Human embryonic kidney HEK-293 cells (ATCC CRL-1573) were obtained from ECACC and were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 100 U/ml penicillin and 100 µM/ml streptomycin.

Human alveolar epithelial cell isolation and culture

Human alveolar type II (ATII) epithelial cells were freshly isolated from non-tumour lung tissue obtained from patients undergoing lung resection. The use of human material was approved by Saarland State Medical Board, Germany. Isolation of ATII was performed according to a protocol adapted from Ehrhardt *et al.* [18]. Purified ATII cells were seeded at a density of 600,000 cells/cm² on collagen and fibronectin-coated plastics using complete SAGM medium supplemented with 100 U/ml penicillin, 100 µM/ml streptomycin and 1% FBS. Primary cells were used after transdifferentiation into monolayers of type I-like (ATI-like) phenotype, following 8 days of culture [13].

Cell transfection

Plasmid transfection

The generation of the expression plasmid containing human δ_1 -ENaC cDNA in a pcDNATM 3.1(+) vector is described in detail elsewhere [44]. NCI-H441 and HEK-293 cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) following the manufacturer's protocol. To determine transient δ -ENaC over-expression, protein samples were collected 48 h after transfection, quantified and analysed by Western blot.

Generation of a stably δ -ENaC over-expressing cell line

Forty-eight hours post-transfection with δ_1 -ENaC plasmid, NCI-H441 cells were split into medium containing 600 μ g/ml geneticin (G418, Sigma-Aldrich), and 10 μ M benzamil. The G418 concentration of 600 μ g/ml was previously shown to be suitable for selection. Several G418-resistant colonies were picked, expanded and assessed for δ -ENaC expression by Western blot. Colonies stably expressing δ -ENaC were kept in culture using the geneticin/benzamil supplemented medium, and cells deriving thereof were used for subsequent expression and functional studies.

Western blot

Cell cultures were lysed on ice using cell extraction buffer (Invitrogen) supplemented with protease-inhibitor cocktail (Sigma-Aldrich). Cell lysates were then briefly sonicated and centrifuged (10,000g at 4°C) for 20 min. The protein concentrations of the obtained supernatants were determined using a standard protein assay (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions. Samples were loaded at equal protein concentrations, separated by electrophoresis using 8.5% polyacrylamide gels, and subsequently transferred to immunoblot polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked in phosphate-buffered saline containing 5% BSA and 0.1% Tween

20 (pH 7.4) at 4°C overnight. Incubation with the respective primary antibody for 2 h at room temperature (i.e. Na⁺/K⁺-ATPase; anti-δ-C 1:5,000 and β-actin 1:8,000) was followed by 1 h incubation with HRP-conjugated secondary antibodies at room temperature (at 1:12,500 dilution). Peroxidase activity was detected with Immobilon Western Chemiluminescent HRP substrate (Millipore). Relative levels of protein expression were quantified by densitometric analysis of the immunoblot using a ChemiDoc documentation system (Bio-Rad). To ensure equal loading, protein was normalised to β-actin. Protein samples obtained from wild-type and mock-transfected HEK-293 cells were used as negative controls. Positive controls were lysates from HEK-293 cells transiently transfected with the full-length δ₁-ENaC plasmid.

Biotinylation of cell surface proteins

Confluent, Transwell-grown cell monolayers were washed with ice-cold PBS containing 2.0 mM CaCl₂. Sulfo-NHS-biotin (0.5 mg/ml, Thermo Scientific Pierce, Medical Supply Company, Dublin, Ireland) was then applied to the apical or basolateral compartments of the monolayers. Phosphate-buffered saline supplemented with 10% BSA and 2.0 mM CaCl₂ was added to the respective opposite compartments, to prevent biotinylation of the opposite cell surface. After 20 min incubation at 4°C, 10% BSA in PBS was added to both compartments to stop the biotinylation reaction, and cell monolayers were incubated for another 20 min. Following cell lysis, protein concentrations of the whole cell lysates were determined by Bradford assay and equal amounts of protein were loaded onto streptavidin-agarose beads (500 µg whole cell protein/100 µl streptavidin-agarose beads) for overnight incubation at 4°C. The next day, biotinylated membrane proteins, now bound onto beads, were separated from the non-biotinylated proteins by centrifugation, washed, resuspended in protein loading buffer and used for Western blot.

Protein deglycosylation

GlycoProfile II, Enzymatic In-Solution N-Deglycosylation kit (PP0201, Sigma-Aldrich) was used according to manufacturer's instructions. Briefly, protein samples were denatured by incubation with denaturant solution containing 2% octyl- β -D-glucopyranoside and 100 mM β -mercaptoethanol at 100°C for 10 min and diluted to the final protein concentration of 1 mg/ml. Peptide N-glycosidase F (PNGase F) was added at the concentration of 50 units/ml. Equal volumes of water were added to the untreated control samples. After 6 h of incubation at 37°C, the reaction was stopped by heating at 100°C for 10 min. Aliquots of PNGase F treated samples and untreated controls were used for subsequent analysis by Western blot.

Immunofluorescence microscopy (IFM)

Transwell-grown cell monolayers were fixed by 10 min incubation with ice-cold methanol, followed by permeabilisation with 0.1% Triton X-100 for 8 min. Unspecific binding sites were blocked by incubation with PBS containing 5% BSA for 30 min at 37°C. After 60 min incubation at 37°C with a 1:50 dilution of the anti- δ -ENaC antibody in PBS containing 1% BSA, the cell layers were washed three times with PBS (1% BSA). Subsequently, cells were incubated for 30 min at 37°C with a 1:1000 dilution of Alexa Fluor-488-labelled F(ab')₂ fragment in PBS containing 1% BSA. Nuclei were counterstained with propidium iodide (1 μ g/ml in PBS). The specimens were then washed three times with PBS (1% BSA) and embedded in FluorSave anti-fade medium (Merck, Nottingham, UK). Images were obtained using a confocal laser scanning microscope (CLSM, Zeiss LSM 510, Göttingen, Germany). The same pinhole settings and the detector gains were used to detect and compare the signals obtained in the wild-type and δ -ENaC over-expressing NCI-H441 cells. Cell monolayers stained with the secondary antibodies only were used as background controls.

Ussing chamber studies

Transwell-grown cell monolayers were regarded as suitable for the transepithelial measurements when the net TEER values exceeded 300 Ω cm². Confluent cell monolayers were mounted into modified Ussing chambers. Krebs-Ringer-Buffer pH 7.4 was added to both compartments of the chamber (composition of KRB: 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH₂PO₄, 25 mM NaHCO₃, 5.55 mM glucose, 15 mM HEPES, 1.8 mM CaCl₂, 0.81 mM MgSO₄). Sodium-free solutions contained equivalent amounts of KCl, KH₂PO₄ and KHCO₃, respectively, whereas in Cl⁻-free solutions, equivalent amounts of the relevant gluconate salts were used.

Chambers and bathing solutions were maintained at 37°C and continuously stirred. The transepithelial potential difference (PD) was clamped to 0.0 mV using a standard four-electrode voltage clamp (DVC-1000, World Precision Instruments, Sarasota, FL), and the short-circuit current (I_{sc}) was continuously recorded using Chart5 for Windows (ADInstruments, Oxfordshire, UK). Voltage impulses of 0.5 mV were given every 50 s in order to monitor the TEER of cell monolayers. Cell monolayers were allowed to reach steady state basal I_{sc} before the compound of interest was added. The used stock solutions were 50 mM Evans blue, 5 mM capsaizepine, 10 mM icilin, 2.5 mM CFTR(inh)₁₇₂, 50 mM amiloride and 100 mM ouabain (all in DMSO; all from Sigma-Aldrich).

Statistical analysis

All experiments were carried out at least in triplicate using cells from at least three different passages. Results, all given as means $\pm$ SD, were compared using one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls post-hoc test. $P < 0.05$ was considered significant.

RESULTS

Expression of δ -ENaC in human respiratory epithelial cells

Immunoblots from Calu-3, NCI-H441 and ATI-like cell lysates as well as negative and positive controls were probed with two different antibodies, i.e. anti- δ -N and anti- δ -C. The anti- δ -N antibody revealed identical band patterns for non-transfected, mock-transfected and δ_1 -ENaC over-expressing HEK-293 cells (*Supplementary Material Figure S1*). Due to this unreliability, further studies were carried out using only the anti- δ -C antibody.

An unspecific band at 100 kDa was observed in all cell lysates (Figure 1a). Nevertheless, a strong band at approximately 70-85 kDa was found in the δ_1 -ENaC over-expressing HEK-293 cell lysates. This band possibly represented merged double bands of δ -ENaC protein at 75 kDa and its glycosylated form at 86 kDa [23]. In lysates from Calu-3, NCI-H441 and ATI-like cells, multiple protein bands were observed, at 200 (Calu-3 only), 100 and 75 kDa as well as further protein bands at lower molecular weights. The band at 75 kDa was weak but corresponded to the δ_1 -ENaC band in over-expressing HEK-293 cells. The presence of this band in all three respiratory epithelial cell types tested confirmed δ -ENaC protein synthesis in these cells.

Localisation of δ -ENaC in human respiratory epithelial cells

Immunofluorescence images showing the subcellular localisation of the δ -ENaC protein are depicted in Figure 1b-d. Transepithelial electrical resistance values, measured prior to fixation and permeabilisation procedure, were $> 300 \Omega\text{cm}^2$ in all cases. Additionally, staining for Na^+/K^+ -ATPase, as a basolateral marker, and for the tight-junctional protein, ZO-1, confirmed polarised cell monolayers with well-developed cellular contacts (data not shown).

Immunofluorescence micrographs showed a mainly intracellular protein localisation. The punctate character of the staining suggests vesicular localisation of the detected protein. The localisation pattern of the signal was different in NCI-H441 and ATI-like *versus* Calu-3 cells.

The signal was predominantly perinuclear in NCI-H441 and ATI-like cells, but accumulated primarily along the cell borders in Calu-3 cells.

Effects of modulators of δ -ENaC activity in human respiratory epithelial cells

We used a pharmacological approach to test for functional δ -ENaC activity in human respiratory epithelium *in vitro*. The δ -ENaC inhibitor, Evans blue (EB), was added to the apical side of the Ussing chamber and increased basal I_{SC} across Calu-3 cell monolayers (by $116.8 \pm 36.2\%$ (100 μ M) and $269.1 \pm 114.5\%$ (300 μ M), respectively (Figure 2a, c)). In contrast, in NCI-H441 cell monolayers, EB decreased I_{SC} in a concentration-dependent manner by $56.4 \pm 11.9\%$ (100 μ M) and $70.0 \pm 14.9\%$ (300 μ M) of basal I_{SC} (Figure 2b, c). ATI-like primary cell monolayers responded similarly to EB, i.e. $62.5 \pm 10.0\%$ and $76.3 \pm 4.2\%$ of the basal I_{SC} were blocked by 100 and 300 μ M EB (Figure 2b, c). After removal of the EB-containing solution, short-circuit currents returned to basal levels (data not shown). The δ -ENaC activator, capsazepine inhibited basal I_{SC} in a concentration-dependent manner in all three cell types tested (Figure 3a, b, c). The inhibition reached a maximum of $40.3 \pm 11.5\%$ in Calu-3, 29.4 ± 6.4 in NCI-H441 and $43.5 \pm 21.3\%$ in ATI-like cells.

Apical administration of the δ -ENaC activator, icilin led to a concentration-dependent increase in I_{SC} in Calu-3 cell monolayers with current activation of $79.0 \pm 29.4\%$ by 60 μ M icilin (Figure 3d, f). Inhibitory effects of icilin of 20.3 ± 5.6 and $43.6 \pm 12.8\%$ on I_{SC} were observed in NCI-H441 and ATI-like cells, respectively (Figure 3e, f).

Ion dependencies of the effects, induced by δ -ENaC modulators in human respiratory epithelial cells

We determined whether effects caused by the δ -ENaC modulators were dependent on sodium or chloride transport. Figure 4a summarises the impact of sodium removal and the inhibition of amiloride-sensitive channels, on the short circuit current in NCI-H441 cell monolayers.

Basal I_{SC} was significantly reduced from $8.8 \pm 3.3 \mu A/cm^2$ to $0.1 \pm 0.6 \mu A/cm^2$ following sodium removal from the bathing solution. Addition of amiloride ($10 \mu M$) also abolished I_{SC} to $0.8 \pm 0.4 \mu A/cm^2$. EB ($300 \mu M$) significantly decreased I_{SC} from 8.2 ± 3.5 to $2.4 \pm 1.4 \mu A/cm^2$. Addition of $10 \mu M$ amiloride led to a further significant reduction to $0.5 \pm 0.5 \mu A/cm^2$. I_{SC} values measured after application of both EB and amiloride were not significantly different from values obtained following addition of amiloride alone. In sodium-free bathing solution, administration of EB did not have a significant effect on I_{SC} . Capsazepine at $20 \mu M$ decreased I_{SC} from 7.6 ± 3.5 to $5.1 \pm 2.5 \mu A/cm^2$. Addition of $10 \mu M$ amiloride reduced the I_{SC} further to $0.3 \pm 0.2 \mu A/cm^2$. In the absence of sodium, no significant capsazepine effect was observed. Icilin produced a fall in I_{SC} from 9.0 ± 4.2 to $7.2 \pm 3.2 \mu A/cm^2$ at $60 \mu M$ but failed to produce a significant effect in sodium-free KRB. When amiloride was added after icilin, an additional inhibition of short-circuit current was observed. Again, the resulting values were not significantly different from the I_{SC} inhibited by amiloride alone. In summary, these measurements indicate that EB, capsazepine and icilin exerted inhibitory effects on amiloride-sensitive sodium currents in NCI-H441 cells.

In Calu-3 cell monolayers, effects induced by these δ -ENaC modulators were also sodium-dependent, but not amiloride-sensitive (data not shown). To test whether CFTR and chloride transport play a role in the observed effects of EB, capsazepine and icilin, measurements were performed in chloride-free conditions and in the presence of the CFTR inhibitor, CFTR(inh)₁₇₂ (Figure 4b). Removal of chloride ions led to a significant decrease of I_{SC} from 6.2 ± 1.6 to $1.9 \pm 2.8 \mu A/cm^2$. Moreover, $69.8 \pm 16.7\%$ of the basal I_{SC} was blocked by addition of $10 \mu M$ of the CFTR channel inhibitor, CFTR(inh)₁₇₂. Short-circuit currents after application of CFTR(inh)₁₇₂ were not significantly different from those measured in chloride-free KRB. EB significantly increased I_{SC} from 6.9 ± 2.4 to $14.9 \pm 3.7 \mu A/cm^2$ (measured 15 min after adding the compound). In chloride-free medium, no significant activation of I_{SC} by EB was observed.

The presence of CFTR(inh)₁₇₂ did not abolish the increase in current caused by EB. When added after CFTR(inh)₁₇₂, EB increased the I_{SC} to $16.8 \pm 1.0 \mu\text{A}/\text{cm}^2$. Both the resulting I_{SC} and the amplitude of the current activation were not significantly different from the values in the absence of CFTR(inh)₁₇₂. When CFTR(inh)₁₇₂ was added after EB, I_{SC} values decreased to $10.2 \pm 2.8 \mu\text{A}/\text{cm}^2$. After removal of chloride ions from the bathing solution, capsazepine led to a decrease in I_{SC} from 0.2 ± 1.4 to $-1.9 \pm 1.3 \mu\text{A}/\text{cm}^2$. The resulting I_{SC} was significantly different from the I_{SC} in response to capsazepine in complete KRB, which was due to significantly different basal currents. The amplitudes of the responses, however, were not significantly different. CFTR(inh)₁₇₂ decreased the I_{SC} by the same amplitude in the presence and absence of capsazepine ($-3.6 \pm 0.6 \mu\text{A}/\text{cm}^2$ and $-4.2 \pm 1.1 \mu\text{A}/\text{cm}^2$, respectively). Capsazepine, added in the presence of CFTR(inh)₁₇₂, led to a further decrease in I_{SC} from 1.3 ± 0.5 to $0.1 \pm 0.6 \mu\text{A}/\text{cm}^2$. The I_{SC} levels were elevated by 60 μM icilin from $7.2 \pm 2.2 \mu\text{A}/\text{cm}^2$ to $12.6 \pm 3.1 \mu\text{A}/\text{cm}^2$ under standard conditions, measured 8 min after adding the compound. In contrast, icilin decreased the I_{SC} in chloride-free medium (from 4.4 ± 2.5 to $0.4 \pm 3.9 \mu\text{A}/\text{cm}^2$). In the presence of CFTR(inh)₁₇₂, icilin increased I_{SC} from 1.3 ± 0.9 to $3.1 \pm 1.3 \mu\text{A}/\text{cm}^2$. The resulting I_{SC}, however, as well as the amplitude of the increase were significantly lower than in the absence of the CFTR-inhibitor ($\Delta I_{SC} = 1.8 \pm 1.3 \mu\text{A}/\text{cm}^2$ and $5.4 \pm 1.8 \mu\text{A}/\text{cm}^2$ in the presence and absence of CFTR-inhibitor, respectively). Furthermore, when added after icilin, CFTR(inh)₁₇₂ completely abolished the increase induced by icilin. The resulting I_{SC} of $2.9 \pm 0.1 \mu\text{A}/\text{cm}^2$ was significantly smaller than the basal current in Calu-3 cells and of similar magnitude compared to the I_{SC} levels in the presence of CFTR-inhibitor alone ($2.0 \pm 1.3 \mu\text{A}/\text{cm}^2$). In summary, the obtained results suggested that the currents activated by EB and icilin in Calu-3 cells were chloride-dependent. Nevertheless, while the effects of icilin appeared as mediated by CFTR, the effect of EB was CFTR-independent. The decrease in I_{SC} induced by capsazepine was both chloride and CFTR-independent.

Knockdown and over-expression of δ -ENaC in human respiratory epithelial cells

In order to test whether the stimulatory/inhibitory effects observed with the alleged δ -ENaC modulators were the result of a specific and exclusive interaction with δ -ENaC, RNAi technique was used to transiently (using siRNA) and stably (using shRNA) transfect NCI-H441 and Calu-3 cells to down-regulate expression of δ -ENaC (*see Supplementary Material*). However, neither RNAi technique resulted in a significant further decrease of δ -ENaC protein (from already low levels at baseline) (*Supplementary Material Figure S2*). Moreover, effects of the δ -ENaC modulators on the currents in transfected Calu-3 and NCI-H441 cells were comparable to those in the respective non-transfected and mock-transfected cells (*Supplementary Material Figure S3*).

Expression and localisation of δ_1 -ENaC protein over-expressed in NCI-H441 cells

In cell lysates from δ -ENaC over-expressing NCI-H441 cells, a prominent protein band was detected at 75 kDa. In addition, a band at 70 kDa, only occasionally observed in wild-type NCI-H441 cells, appeared consistently in all samples from transfected cells (Figure 5a-c). Following PNGase F treatment, the main band shifted from 75 kDa to 70 kDa (Figure 5d), attesting that these bands represented *N*-glycosylated and non-glycosylated forms of the over-expressed δ_1 -ENaC protein.

To study the localisation of the over-expressed δ_1 -ENaC protein, NCI-H441 cells were grown on Transwell inserts and immunostained using the anti- δ -C antibody. Higher intensity as well as clearly changed distribution pattern of the fluorescent signal was observed in the δ_1 -ENaC over-expressing (Figure 6b) compared to that in the wild-type cells (Figure 6a). A strong accumulation of the fluorescent signal for the over-expressed δ -ENaC was observed at the apical surfaces of the transfected cells. To further confirm the localisation of δ -ENaC protein, apical and basolateral surface proteins of confluent NCI-H441 cell monolayers grown on Transwell inserts were biotinylated and analysed by Western blot. No δ -ENaC protein was

detected in the biotinylated membrane protein fractions of wild-type cells (Figure 6c). Similar observations were made for ATI-like cell monolayers (data not shown). In apical and basolateral membrane fractions of Calu-3 cells, a band at 36 kDa was visible (data not shown). In contrast, the *N*-glycosylated 75 kDa form of δ -ENaC was detected on both cellular aspects of the transfected cells. The non-glycosylated form of 70 kDa was only observed in the basolateral membrane fraction (Figure 6d). This discrepancy may, however, be an artefact originating from less efficient recovery of the apically biotinylated protein samples.

Amiloride-sensitivity of sodium currents in δ_1 -ENaC over-expressing NCI-H441 cells

The effect on the sodium currents caused by amiloride in δ_1 -ENaC over-expressing NCI-H441 cells differed significantly from what was observed in wild-type cells. Representative current traces obtained upon consecutive administration of 10 and 100 μ M amiloride as well as the quantification of the effects are shown in Figure 7. In the transfected NCI-H441 cells, administration of 10 μ M amiloride significantly reduced basal I_{SC} by $26.3 \pm 10.1\%$. The effect was significantly smaller than in the wild-type cells, where $88.3 \pm 5.9\%$ of basal I_{SC} were inhibited by 10 μ M amiloride. At 100 μ M, amiloride led to a further significant decrease in current to $20.3 \pm 1.1\%$ of the initial value in the transfected and to $3.8 \pm 4.0\%$ in the non-transfected cells. Inhibition of Na^+/K^+ -ATPase by ouabain (1 mM) led to a similar reduction of I_{SC} in both cell types (by $78.5 \pm 19.4\%$ and $69.7 \pm 5.2\%$ in the wild-type and transfected cells, respectively; Figure 7c).

Effects of δ -ENaC modulators on the I_{SC} in δ_1 -ENaC over-expressing NCI-H441 cells

Evans blue, capsazepine and icilin showed pronounced effects on I_{SC} in δ_1 -ENaC over-expressing NCI-H441 cells. In Figure 8d, e and f representative current traces in response to EB (300 μ M), capsazepine (20 μ M) and icilin (60 μ M) are shown. EB led to a significant reduction of I_{SC} , which was comparable to data obtained in non-transfected cells (i.e. a

decrease by $70.1 \pm 19.1\%$ and $70.0 \pm 14.9\%$, respectively; Figure 9). Both activators of δ -ENaC function, capsazepine and icilin, augmented I_{SC} levels in δ_1 -ENaC over-expressing NCI-H441 cells (significant activation by $33.0 \pm 7.7\%$ and $30.4 \pm 10.1\%$, respectively), contrary to their inhibitory effect exhibited in wild-type cells (Figure 9). In summary, the effects in δ -ENaC over-expressing NCI-H441 cells were consistent with the original observation on the compounds using *Xenopus laevis* oocytes [46-48].

Amiloride-sensitivity of the effects induced by δ -ENaC modulators in δ_1 -ENaC over-expressing NCI-H441 cells

Figure 10a shows the effects of 10 and 100 μ M amiloride on the I_{SC} of δ_1 -ENaC over-expressing NCI-H441 cell monolayers, which have previously been treated with EB (300 μ M), capsazepine (20 μ M) or icilin (60 μ M). Short-circuit currents inhibited by EB ($28.9 \pm 16.3\%$ of basal I_{SC} values) were slightly decreased by the addition of 10 and 100 μ M amiloride ($24.3 \pm 18.6\%$ and $20.8 \pm 18.2\%$ of basal I_{SC} , respectively). Capsazepine and icilin-induced increases in I_{SC} (by $34.4 \pm 8.4\%$ and $31.3 \pm 11.8\%$ of basal I_{SC} , respectively) were completely abolished by the administration of 10 μ M amiloride ($90.6 \pm 8.2\%$ and $72.6 \pm 6.7\%$ of the basal I_{SC} , respectively). Subsequent increase to 100 μ M amiloride led to a further significant decrease in I_{SC} ($49.4 \pm 18.6\%$ and $21.5 \pm 14.5\%$ of the basal I_{SC} , respectively). On the other hand, addition of capsazepine or icilin significantly stimulated currents across cell monolayers pre-treated with 10 μ M amiloride (by $11.3 \pm 5.2\%$ and $12.5 \pm 2.6\%$ of the amiloride-insensitive I_{SC} , respectively), albeit the amplitudes of the increases were significantly smaller than in the absence of amiloride (Figure 10b).

Evans blue-sensitivity of the effects induced by δ -ENaC activators in δ_1 -ENaC over-expressing NCI-H441 cells

Capsazepine-induced current increases ($30.2 \pm 6.4\%$ of basal I_{SC}) were completely abolished by application of EB ($300 \mu\text{M}$). The resulting value (i.e. $10.6 \pm 12.1\%$ of basal I_{SC}) was not significantly different from I_{SC} in the presence of EB alone ($29.9 \pm 19.1\%$). Furthermore, capsazepine failed to activate currents in EB pre-treated cell monolayers. Representative I_{SC} traces and quantification of the effects are shown in Figure 11a, b and e.

Similar results were obtained with icilin (Figure 11c-e). In the presence of EB, no significant current stimulation by icilin was observed. On the contrary, icilin further reduced I_{SC} levels already diminished by EB (from $24.2 \pm 17.2\%$ to $20.2 \pm 18.0\%$ of the basal I_{SC}), albeit the effect did not reach significance. Currents initially activated by icilin ($28.5 \pm 7.1\%$) were significantly reduced by EB. The resulting I_{SC} value (i.e. $11.3 \pm 4.6\%$ of basal I_{SC}) was not significantly different from I_{SC} in the presence of EB alone ($29.9 \pm 19.1\%$).

DISCUSSION

There is a growing body of evidence confirming the presence of the δ -ENaC subunit in human respiratory epithelium. Delta-ENaC mRNA transcripts and protein have been detected in various continuously growing respiratory epithelial cell lines (i.e. A549, NCI-H441 and 16HBE14o-), freshly isolated respiratory epithelial cells in primary culture (ATI, ATII, HNE) and human lung tissue *in situ* [3, 27, 37, 50]. As a functional role of δ -ENaC in these epithelia, contribution to transepithelial sodium transport in human alveolar epithelium in health and disease has been suggested [28]. However, as with most other postulates regarding δ -ENaC function, this hypothesis is based on functional data acquired in δ -ENaC expressing *X. laevis* oocytes [27]. Supportive evidence in organotypic models is limited to two reports [3, 40]. The absence of δ -ENaC gene expression in rodents, where it is probably a pseudogene [21], hinders the development of experimental animal models to study the physiological relevance of δ -ENaC in a more complex system.

In this work, organotypic cell models of respiratory epithelium, commonly employed to investigate pulmonary solute and ion transport in the airways, were used to study the functional expression of δ -ENaC *in vitro*, i.e. NCI-H441 and Calu-3 cell lines as well as the human alveolar epithelial type I-like cells in primary culture. Whilst both δ -ENaC mRNA and protein have been detected in NCI-H441 cells and human lung tissue [27, 37, 50], δ -ENaC expression in Calu-3 bronchial epithelial cells has not been yet reported.

Delta-ENaC expression and localisation

In the literature, distinct molecular weights have been reported for the δ -ENaC protein detected by Western blot. The observed differences appear to depend on the type of the experimental model used. In particular, the protein processing, cleavage and glycosylation may differ between endogenously and heterologously expressed proteins. Moreover, differences between various expression systems, i.e. *X. laevis* oocytes *versus* HEK-293 cells,

and organ systems, i.e. the skin *versus* the lung, may not be excluded. For the endogenous δ -ENaC, a single protein band was observed in samples from human skin and keratinocytes (~70 kDa, [49] as well as mouse sperm (~80 kDa, [24], whilst a double band at 80-90 kDa was detected in the lysates of human bronchiolar epithelial NCI-H441 and pleural mesothelial M9K cells [38] as well as in the human glioma D54-MG cell line [30]. Also, for heterologously expressed δ -ENaC, products of various molecular sizes have been reported. In CHO cells, a single 70 kDa δ -ENaC protein was observed [49]. In COS-7 cells, two δ -ENaC-specific bands at 85 and 95 kDa appeared, with the upper band shifting downwards to 90 kDa following the PNGase F treatment [12]. In *X. laevis* oocytes expressing δ -ENaC, on the other hand, [23] detected two prominent bands at 75 and 86 kDa, representing an *N*-glycosylated and a non-glycosylated form of the same protein.

With the anti- δ -C antibody, a protein of approximately 75 kDa was detected in lysates of HEK-293 cells, heterologously expressing δ -ENaC. Faint bands at the corresponding molecular weight were also observed in the whole cell lysates of Calu-3, NCI-H441 and ATI-like cells. Unexpectedly, only low δ -ENaC signals were detected in cell membranes by means of the immunofluorescence microscopy with either of the antibodies, revealing an intracellular, most likely vesicular localisation of δ -ENaC protein in NCI-H441, Calu-3 and ATI-like cells. Cell surface biotinylation confirmed the absence of δ -ENaC from the cell surface of NCI-H441 cells.

Effects of pharmacological modulators of δ -ENaC function on the I_{SC} in human respiratory epithelial cells in vitro

Three substances have been reported to be able to extract δ -ENaC-mediated currents from the $\alpha\beta\gamma$ -ENaC-mediated sodium transport. Evans blue ($IC_{50} = 143 \mu M$), capsazepine and icilin ($EC_{50} = 8 \mu M$ and $33 \mu M$, respectively) inhibited/stimulated δ -ENaC-activity in *X. laevis* oocytes expression system, without having an impact on α -ENaC [46-48]. Only a single

publication, however, reported the use of these compounds as modulators of δ -ENaC function in an organotypic *in vitro* model, i.e. in human nasal epithelial (HNE) cells [3]. No functional evidence has yet been provided for other respiratory epithelial cell types, in particular NCI-H441 cells, for which δ -ENaC expression has been reported repeatedly [27, 37, 38].

In our hands, Evans blue reduced currents across both NCI-H441 and ATI-like cell monolayers, which was consistent with previous observations in oocytes [48] and HNE cells [3], but exhibited concentration-dependent stimulatory effects on the I_{SC} in Calu-3 cells.

Capsazepine, on the other hand, decreased the I_{SC} in all cell types tested, albeit being suggested as a δ -ENaC activator [46]. Icilin, another reported activator [47], caused a concentration-dependent I_{SC} increase in Calu-3 cell monolayers, but exhibited inhibitory effects in NCI-H441 and ATI-like cell monolayers.

All three substances are known to modulate targets other than δ -ENaC *in vitro* and *in situ*.

Capsazepine, for example acts as a competitive antagonist on both TRPV1 and TRPM8 receptors [6, 7], whereas icilin as a “super-cooling agent” activates TRPM8 [2]. Expression of both TRPV1 and TRPM8 receptors has been reported in human respiratory epithelial cells, so that their regulation by capsazepine and icilin may account for the outcomes observed with these compounds. Hence, despite the absence of direct evidence, it is conceivable that these ion channels may be differentially expressed in bronchial Calu-3, bronchiolar H441 and ATI-like cells, with inhibition of TRPM8 and TRPV1 by capsazepine and activation of TRPM8 by icilin leading to the respective inhibitory or stimulatory effects. Moreover, capsazepine also blocks voltage-dependent potassium channels [31]. Expression of the latter has been reported in rat alveolar epithelial cells in primary culture and in Calu-3 cells with localisation at the apical cell surfaces [32], offering an alternative explanation for the observed capsazepine-induced I_{SC} decreases in Calu-3, H441 and ATI-like cell monolayers.

Among the substances tested, EB is probably the least specific compound, since it has been reported to interact with a range of different proteins. Besides δ -ENaC, it inhibits P_2X -

purinergic receptors and glutamate transporters [9]. Activation of large-conductance, Ca^{2+} activated K^+ channels (BK) is another mode of action exhibited by EB [25]. Functional BK channels have been most recently described at the apical surfaces of NHBE cells [35]. Importantly, not only did activation of the latter stimulate the apical K^+ secretion in NHBE cells, it also triggered an increase in apical Cl^- efflux, suggesting that current stimulation by EB in Calu-3 cells may indeed occur via BK channels.

Ultimately, it is beyond the scope of this work to identify the exact molecular pathways leading to the observed, pharmacological effects. Nevertheless, it is noteworthy to highlight the potential relevance of further investigating the nature of EB-induced increases in I_{SC} in Calu-3 cells, as the underlying mechanism could serve as a novel therapeutic approach in the treatment of cystic fibrosis.

Delta-ENaC over-expression studies

Delta-ENaC was stably over-expressed in NCI-H441 cells. The specific δ -ENaC protein band at 75 kDa was of a significantly higher intensity in the transfected cells, and was demonstrated to be an *N*-glycosylated form of a 70 kDa protein, which was consistent with the previous reports of δ -ENaC protein being *N*-glycosylated [12, 23]. Moreover, elevated protein levels led to an increased insertion into the apical cell membrane, as confirmed by IFM. Conflicting data generated by surface biotinylation, which showed the over-expressed protein on both membranes, is likely an artefact [22].

In the Ussing chamber studies, sodium currents across δ -ENaC over-expressing cell monolayers displayed significantly lower sensitivities to amiloride than in the wild-type cells. Expectedly, EB exhibited inhibitory effects on the I_{SC} in δ -ENaC over-expressing cells. Capsazepine and icilin, on the other hand, increased the I_{SC} levels, in agreement with the characterisation of both compounds as δ -ENaC activators [46-47] and opposed to the current reduction observed with both substances in the wild-type cells. Highly selective sodium

channels (HSC), with a large selectivity for sodium over potassium, poorly selective channels displaying moderate selectivity as well as the non-selective cation channels (NSC) have been defined in the alveolar epithelial cells by single channel measurements and are responsible for the alveolar transepithelial sodium transport [17]. While the classic $\alpha\beta\gamma$ -ENaC is believed to represent the HSC, the molecular basis of the two other groups has remained controversial, with homomeric α -ENaC channels, $\alpha\beta$ - or $\alpha\gamma$ -ENaC channels as well as different combinations of subunits with δ -ENaC being the potential candidates [17, 27]. Distinct affinities to the ENaC blocker amiloride also account for biophysical differences between the different channel types. In NCI-H441 cells, the presence of two populations of Na^+ channels (HSC and NSC) has been described, with $\sim 55\%$ of total I_{SC} being sensitive to $1\ \mu\text{M}$ amiloride, further 30% and 5% being inhibited by 10 and $100\ \mu\text{M}$ amiloride, respectively [1]. These observations are consistent with the amiloride-sensitivity of the wild-type NCI-H441 cells in our hands, where $10\ \mu\text{M}$ amiloride was sufficient to inhibit the main portion of the I_{SC} ($\sim 90\%$), and merely $\sim 5\%$ of the basal currents showed additional sensitivity to $100\ \mu\text{M}$ amiloride. In δ_1 -ENaC over-expressing NCI-H441 cells, on the other hand, only $\sim 25\%$ of the basal I_{SC} were inhibited by addition of $10\ \mu\text{M}$ amiloride. An additional 55% were sensitive to $100\ \mu\text{M}$ amiloride. Importantly, the contribution of sodium currents to the total I_{SC} was similar in both cell types, as demonstrated by inhibition of Na^+/K^+ -ATPase by ouabain. These results suggest that the amiloride-refractory NSC channels account for a larger portion of the total sodium current and thus, for a larger portion of the surface channel population in the transfected NCI-H441 cells.

Moreover, currents stimulated by the alleged δ -ENaC activators were, at least partially, insensitive to $10\ \mu\text{M}$ amiloride, since both compounds were able to produce significant I_{SC} increases in the monolayers pre-treated with $10\ \mu\text{M}$ amiloride. Furthermore, the stimulatory effects were completely abolished by application of $100\ \mu\text{M}$ amiloride or $300\ \mu\text{M}$ EB. Thus, the interdependencies of the inhibitors, amiloride and EB, with the activators, capsaizepine and

icilin, were consistent with the assumption that the compounds interacted with the same target, i.e. δ -ENaC.

CONCLUSIONS

This work confirmed δ -ENaC expression in human respiratory epithelial NCI-H441 cell line and human alveolar epithelial cells in primary culture. The presence of δ -ENaC in human bronchial epithelial Calu-3 cell line was described for the first time. However, the abundance of δ -ENaC protein was very low and δ -ENaC protein expression was not detected in the apical cell membrane. More importantly, this study demonstrated that, despite its expression in human respiratory epithelial cells *in vitro*, δ -ENaC function was not evident in the chosen airway epithelial cell models. Thus, the relevant contribution of endogenous δ -ENaC to net transepithelial sodium transport across respiratory epithelium, as suggested by studies in heterologous expression systems, could not be confirmed.

Since our work focused on the transepithelial ion movement under basal, unstimulated conditions, future investigations are required to clarify whether δ -ENaC function can be up-regulated in human airway epithelium. Moreover, based on our findings, the specificity of currently available pharmacological tools for characterisation of δ -ENaC function are highly unsatisfactory due to their interference with multiple transporter targets. Therefore, there is demand for more specific pharmacological modulators of δ -ENaC function that would help specifically dissect the contribution of the δ -subunit to ENaC-mediated sodium transport and elucidate the physiological role of δ -ENaC in the human lung and other tissues.

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FIGURE CAPTIONS

Figure 1. Calu-3, NCI-H441 and ATI-like cell lysates were tested for δ -ENaC expression using polyclonal anti- δ -ENaC antibodies. Lysates of non-transfected (*ctrl*) and mock-transfected (*mock*) HEK-293 cells were used as negative controls; δ_1 -ENaC-transfected (δ_1 -ENaC) HEK-293 cells as positive controls. Immunofluorescence microscopy for δ -ENaC (green) in **(b)** Calu-3, **(c)** NCI-H441 and **(d)** ATI-like cells. **(b')**, **(c')** and **(d')** show the respective cross sections along the green X-Z trace. Cells were grown on Transwell Clear inserts. Cell nuclei were counterstained with propidium iodide (red).

Figure 2. Representative short-circuit current (I_{SC}) traces obtained in response to the subsequent apical administration of 100 μ M and 300 μ M Evans blue (EB) to the monolayers of **(a)** Calu-3 and **(b)** NCI-H441 and ATI-like cells: δ -ENaC inhibitor EB increased I_{SC} across Calu-3 cell monolayers, but had an inhibiting effect on the I_{SC} in NCI-H441 and ATI-like cells. **(c)** Short-circuit current levels were measured 15 min after adding the compound, and normalised by the basal I_{SC} in the absence of EB. Results are expressed as means $\pm$ SD ($n = 3-13$). The statistical significance of the effect is expressed as *, $P < 0.01$, versus the basal I_{SC} , and as #, $P < 0.01$ versus the I_{SC} in response to 100 μ M EB

Figure 3. Representative I_{SC} traces obtained in response to the apical administration of 20 μ M capsazepine to the monolayers of **(a)** Calu-3 and **(b)** NCI-H441 and ATI-like cells: The alleged δ -ENaC activator decreased I_{SC} in human respiratory epithelial cells. Sample current traces in response to 60 μ M icilin in **(d)** Calu-3 and **(e)** NCI-H441 and ATI-like cells: Whilst exhibiting inhibitory effects in NCI-H441 and ATI-like cells, icilin stimulated currents in Calu-3 cell monolayers. Short-circuit current levels were measured and normalised by the basal I_{SC} in the absence of **(c)** capsazepine and **(f)** icilin, respectively. Results are expressed as means $\pm$ SD ($n = 4-15$). The statistical significance of the effect is expressed as *, $P < 0.05$ and **, $P < 0.01$, versus the basal I_{SC}

Figure 4. (a) Sodium-dependence of the apical response to 300 μ M EB, 20 μ M capsazepine and 60 μ M icilin in NCI-H441 cells. Results are expressed as means $\pm$ SD ($n = 3-9$). The statistical significance of the differences is expressed as *, $P < 0.01$ versus the basal I_{SC} in KRB buffer, and the I_{SC} in the presence of the relevant compound, respectively. **(b)** Effects of chloride ion removal as well as of inhibition of CFTR-mediated currents on the apical response to 100 μ M EB, 20 μ M capsazepine and 60 μ M icilin in Calu-3 cells. I_{SC} values were measured 15 min after addition of the compounds. Results are expressed as means $\pm$ SD ($n = 3-20$). The statistical significance of the differences is expressed as *, $P < 0.01$ versus the basal I_{SC} , I_{SC} in the presence of the relevant compound or 10 μ M CFTR(inh)₁₇₂, respectively. ¹: substance was added first; ²: substance was added 30 min after the first compound

Figure 5. Over-expression of δ_1 -ENaC in NCI-H441 cells. **(a)** Representative immunoblots of wild-type (*wt*) and δ_1 -ENaC over-expressing NCI-H441 cells (δ_1 -ENaC), probed with the anti- δ -C antibody. **(b)** And **(c)** show densitometric analysis of the 70 kDa and 75 kDa bands in wild-type and δ_1 -ENaC over-expressing NCI-H441 cells, respectively. **(d)** Glycosylation of δ_1 -ENaC protein, over-expressed in NCI-H441 cells. Untreated control samples were run in parallel to PNGase F treated cell lysates

Figure 6. Localisation of δ -ENaC protein in **(a, c)** NCI-H441(*wt*) and **(b, d)** NCI-H441(δ_1 -ENaC) cells. **(a, b)** Immunofluorescence microscopy for δ -ENaC (green) in NCI-H441(*wt*) and NCI-H441(δ_1 -ENaC) cells, grown on Transwell inserts. Cross sections along the X-Z trace (green) and Y-Z trace (red) are shown above and to the right, respectively. Identical pinhole settings and detector gains were used to detect and compare the signals obtained using the anti- δ -C antibody. Cell nuclei were counterstained with propidium iodide (red). White bars represent 10 μ M. **(c, d)** Representative Western blot of whole cell lysates as well as of

apically and basolaterally biotinylated surface protein fractions, obtained from NCI-H441(*wt*) and (δ_1 -ENaC) cells. Beta-actin and Na⁺/K⁺-ATPase were used as controls to exclude contamination of the biotinylated samples with intracellular non-biotinylated protein and contamination of apical protein fraction with basolateral protein, respectively

Figure 7. Representative I_{SC} traces obtained in response to subsequent administration of 10 μ M and 100 μ M amiloride in the **(a)** wild-type and **(b)** δ_1 -ENaC over-expressing NCI-H441 cells. **(c)** Quantification of the responses to 10 μ M and 100 μ M amiloride as well as to 1 mM ouabain. Short-circuit current values were measured after adding the respective concentration of amiloride or ouabain and normalised against the I_{SC} , measured in the absence of the compounds (I_{SC} basal). Results are expressed as means $\pm$ SD ($n = 3 - 9$). The statistical significance of the differences is expressed as *, $P < 0.05$ and as **, $P < 0.01$ versus the basal I_{SC} , the I_{SC} in the presence of 10 μ M amiloride and versus the respective I_{SC} in the wild-type NCI-H441 cells

Figure 8. Representative I_{SC} traces of the responses to **(a, d)** 300 μ M EB, **(b, e)** 20 μ M capsazepine and **(c, f)** 60 μ M icilin are shown, after the compounds were applied apically to the monolayers of the wild-type **(a, b, and c)** or δ_1 -ENaC over-expressing NCI-H441 cells **(d, e, and f)**. All alleged δ -ENaC modulators exhibited inhibitory effects in the wild-type NCI-H441 cells. In δ_1 -ENaC over-expressing cells, only the δ -ENaC inhibitor, EB, decreased the I_{SC} levels. The reported activators, capsazepine and icilin, had stimulatory effects on the I_{SC}

Figure 9. Quantification of the responses to 300 μ M EB, 20 μ M capsazepine and 60 μ M icilin in the wild-type (*black bars*) and δ_1 -ENaC over-expressing NCI-H441 cell monolayers (*grey bars*). Short-circuit current values were measured after addition of the respective compound and normalised against the I_{SC} , measured in the absence of the substance (I_{SC}

basal). Results are expressed as means $\pm$ SD ($n = 9 - 13$). The statistical significance of the effect is expressed as *, $P < 0.01$ versus the basal I_{SC} and the respective I_{SC} in the wild-type NCI-H441 cells

Figure 10. Amiloride-sensitivity of the effects induced by administration of EB, capsazepine and icilin to monolayers of δ_1 -ENaC over-expressing NCI-H441 cells. **(a)** The I_{SC} values, measured after adding the relevant compound and after subsequent application of 10 μ M and 100 μ M amiloride, respectively, were normalised against I_{SC} , measured in the absence of the substance (I_{SC} basal). Results are expressed as means $\pm$ SD ($n = 6$). The statistical significance of the effects is expressed as **, $P < 0.01$ versus the I_{SC} in the presence of the respective compound or in the presence of the relevant substance and 10 μ M amiloride. **(b)** Capsazepine (20 μ M) or icilin (60 μ M) were added to the cell monolayers in the presence or absence of 10 μ M amiloride. I_{SC} values were measured after adding the relevant compound and normalised against the I_{SC} , measured prior to adding the substance (by basal I_{SC} in the absence of amiloride/*black bars* or by I_{SC} in the presence of 10 μ M amiloride/*grey bars*). Results are expressed as means $\pm$ SD ($n = 3 - 9$). The statistical significance of the effect is expressed as *, $P < 0.05$ and **, $P < 0.01$ versus the basal I_{SC} or the I_{SC} in the presence of 10 μ M amiloride, respectively, and as #, $P < 0.01$ versus the compound effect in the absence of amiloride

Figure 11. Capsazepine- and icilin-induced I_{SC} increases were sensitive to EB in δ_1 -ENaC over-expressing NCI-H441 cell monolayers. Representative I_{SC} traces in response to consecutive administration of the δ -ENaC activator **((a)** 20 μ M capsazepine; **(c)** 60 μ M icilin) and 300 μ M EB and *vice versa* **(c and d, respectively)**. **(e)** I_{SC} values were measured after administration of the respective compound and normalised against the I_{SC} , measured in the absence of the substances (I_{SC} basal). Results are expressed as means $\pm$ SD ($n = 3$). The statistical significance of the effect is expressed as n.s., non-significant and as *, $P < 0.01$

versus the I_{SC} in the presence of capsazepine, icilin or EB. ¹: substance was added first; ²: substance was added 10 min after the first compound

Figure 1

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REFERENCES

1. Albert AP, Woollhead AM, Mace OJ, Baines DL (2008) AICAR decreases the activity of two distinct amiloride-sensitive Na^+ -permeable channels in H441 human lung epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 295(5):L837-48. doi: 10.1152/ajplung.90353.2008

2. Andersson DA, Chase HW, Bevan S (2004) TRPM8 activation by menthol, icilin and cold is differentially modulated by intracellular pH. *J Neurosci* 24(23):5364-9. doi: 10.1523/jneurosci.0890-04.2004
3. Bangel-Ruland N, Sobczak K, Christmann T, Kentrup D, Langhorst H, Kusche-Vihrog K, Weber WM (2010) Characterization of the epithelial sodium channel delta-subunit in human nasal epithelium. *Am J Respir Cell Mol Biol* 42(4):498-505. doi: 10.1165/rcmb.2009-0053OC
4. Barker PM, Nguyen MS, Gatzky JT, Grubb B, Norman H, Hummler E, Rossier B, Boucher RC, Koller B (1998) Role of gammaENaC subunit in lung liquid clearance and electrolyte balance in newborn mice. Insights into perinatal adaptation and pseudohypoaldosteronism. *J Clin Invest* 102(8):1634-1640. doi: 10.1172/JCI3971
5. Bastacky J, Lee CY, Goerke J, Koushafar H, Yager D, Kenaga L, Speed TP, Chen Y, Clements JA (1995) Alveolar lining layer is thin and continuous: low-temperature scanning electron microscopy of rat lung. *J Appl Physiol* 79(5):1615-28. **PMID 8594022**
6. Behrendt HJ, Germann T, Gillen C, Hatt H, Jostock R (2004) Characterization of the mouse cold-menthol receptor TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. *Br J Pharmacol* 141(4):737-745. doi: 10.1038/sj.bjp.0705652
7. Bevan S, Hothi S, Hughes G, James IF, Rang HP, Shah K, Walpole CS, Yeats JC (1992) Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br J Pharmacol* 107(2):544-52. **PMID 1422598**
8. Brouard M, Casado M, Djelidi S, Barrandon Y, Farman N (1999) Epithelial sodium channel in human epidermal keratinocytes: expression of its subunits and relation to sodium transport and differentiation. *J Cell Sci* 112 (19):3343-52. **PMID 10504339**

9. Bultmann R, Starke K (1993) Evans blue blocks P₂X-purinoceptors in rat vas deferens. *Naunyn-Schmiedeberg's Arch Pharmacol* 348(6):684-7. **PMID 8133912**
10. Canessa CM, Horisberger JD, Rossier BC (1993) Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature* 361(6411):467-70. doi:10.1038/361467a0
11. Canessa CM, Schild L, Buell G, Thorens B, Gautschi I, Horisberger JD, Rossier BC (1994) Amiloride-sensitive epithelial Na⁺ channel is made of three homologous subunits. *Nature* 367(6462):463-7. doi:10.1038/367463a0
12. Chang T, Ke Y, Ly K, McDonald FJ (2011) COMMD1 regulates the delta epithelial sodium channel (δ ENaC) through trafficking and ubiquitination. *Biochem Biophys Res Commun* 411(3):506-11 2011. doi: 10.1016/j.bbrc.2011.06.149
13. Demling N, Ehrhardt C, Kasper M, Laue M, Knels L, Rieber EP (2006) Promotion of cell adherence and spreading: a novel function of RAGE, the highly selective differentiation marker of human alveolar epithelial type I cells. *Cell Tissue Res* 323(3):475-88. doi: 10.1007/s00441-005-0069-0
14. Dobbs LG, Johnson MD (2007) Alveolar epithelial transport in the adult lung. *Respir Physiol Neurobiol* 159(3):283-300. doi: 10.1016/j.resp.2007.06.011
15. Docherty RJ, Yeats JC, Piper AS (1997) Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurones in culture. *Br J Pharmacol* 121(7):1461-1467. doi: 10.1038/sj.bjp.0701272
16. Duc C, Farman N, Canessa CM, Bonvalet JP, Rossier BC (1994) Cell-specific expression of epithelial sodium channel alpha, beta, and gamma subunits in aldosterone-responsive epithelia from the rat: localization by in situ hybridization and immunocytochemistry. *J Cell Biol* 127(6):1907-21. **PMID 7806569**

17. Eaton DC, Chen J, Ramosevac S, Matalon S, Jain L (2004) Regulation of Na⁺ channels in lung alveolar type II epithelial cells. *Proc Am Thorac Soc* 1(1):10-6. **PMID 16113405**
18. Ehrhardt C, Kim KJ, Lehr CM (2005) Isolation and culture of human alveolar epithelial cells. *Methods Mol Med* 107:207-16. **PMID 15492374**
19. Elias N, Rafii B, Rahman M, Otulakowski G, Cutz E, O'Brodovich H (2007) The role of alpha-, beta-, and gamma-ENaC subunits in distal lung epithelial fluid absorption induced by pulmonary edema fluid. *Am J Physiol Lung Cell Mol Physiol* 293(3):L537-45. doi: 10.1152/ajplung.00373.2006
20. Giraldez T, Afonso-Oramas D, Cruz-Muros I, Garcia-Marin V, Pagel P, González-Hernández T, Alvarez de la Rosa D (2007) Cloning and functional expression of a new epithelial sodium channel delta subunit isoform differentially expressed in neurons of the human and monkey telencephalon. *J Neurochem* 102(4):1304-15. doi: 10.1111/j.1471-4159.2007.04622.x
21. Giraldez T, Rojas P, Jou J, Flores C, Alvarez de la Rosa D (2012) The epithelial sodium channel δ -subunit: new notes for an old song. *Am J Physiol Renal Physiol* 303(3):F328-38. doi: 10.1152/ajprenal.00116.2012
22. Gottardi CJ, Dunbar LA, Caplan MJ (1995) Biotinylation and assessment of membrane polarity: caveats and methodological concerns. *Am J Physiol* 268(2):F285-95. **PMID 7864168**
23. Haerteis S, Krueger B, Korbmacher C, Rauh R (2009) The delta-subunit of the epithelial sodium channel (ENaC) enhances channel activity and alters proteolytic ENaC activation. *J Biol Chem* 284(42):29024-40. doi: 10.1074/jbc.M109.018945

- 24.** Hernández-González EO, Sosnik J, Edwards J, Acevedo JJ, Mendoza-Lujambio I, López-González I, Demarco I, Wertheimer E, Darszon A, Visconti PE (2006) Sodium and epithelial sodium channels participate in the regulation of the capacitation-associated hyperpolarization in mouse sperm. *J Biol Chem* 281(9):5623-33. doi: 10.1074/jbc.M508172200
- 25.** Hollywood MA, Cotton KD, McHale NG, Thornbury KD (1998) Enhancement of Ca^{2+} -dependent outward current in sheep bladder myocytes by evans blue dye. *Pflugers Arch* 435(5):631-6. doi: 10.1007/s004240050563
- 26.** Hummler E, Barker P, Gatzky J, Beermann F, Verdumo C, Schmidt A, Boucher R, Rossier BC (1996) Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nat Genet* 12(3):325-8. doi:10.1038/ng0396-325
- 27.** Ji HL, Su XF, Kedar S, Li J, Barbry P, Smith PR, Matalon S, Benos DJ (2006) Delta-subunit confers novel biophysical features to alpha beta gamma-human epithelial sodium channel (ENaC) via a physical interaction. *J Biol Chem* 281(12):8233-41. doi: 10.1074/jbc.M512293200
- 28.** Ji HL, Zhao RZ, Chen ZX, Shetty S, Idell S, Matalon S (2012) δ -ENaC: a novel divergent amiloride-inhibitable sodium channel. *Am J Physiol Lung Cell Mol Physiol* 303(12):L1013-26. doi: 10.1152/ajplung.00206.2012
- 29.** Johnson MD, Widdicombe JH, Allen L, Barbry P, Dobbs LG (2002) Alveolar epithelial type I cells contain transport proteins and transport sodium, supporting an active role for type I cells in regulation of lung liquid homeostasis. *Proc Natl Acad Sci U S A* 99(4):1966-71. doi: 10.1073/pnas.042689399
- 30.** Kapoor N, Bartoszewski R, Qadri YJ, Bebok Z, Bubien JK, Fuller CM, Benos DJ (2009) Knockdown of ASIC1 and epithelial sodium channel subunits inhibits glioblastoma whole

cell current and cell migration. J Biol Chem 284(36):24526-41. doi:
10.1074/jbc.M109.037390

31. Kuenzi FM, Dale N (1996) Effect of capsaicin and analogues on potassium and calcium currents and vanilloid receptors in *Xenopus* embryo spinal neurones. Br J Pharmacol 119(1):81-90. **PMID 8872360**
32. Lee SY, Maniak PJ, Ingbar DH, O'Grady SM (2003) Adult alveolar epithelial cells express multiple subtypes of voltage-gated K⁺ channels that are located in apical membrane. Am J Physiol Cell Physiol 284(6):C1614-24. doi: 10.1152/ajpcell.00429.2002
33. Li T, Folkesson HG (2006) RNA interference for alpha-ENaC inhibits rat lung fluid absorption *in vivo*. Am J Physiol Lung Cell Mol Physiol 290(4):L649-L660. doi: 10.1152/ajplung.00205.2005
34. Liu L, Simon SA (1997) Capsazepine, a vanilloid receptor antagonist, inhibits nicotinic acetylcholine receptors in rat trigeminal ganglia. Neurosci Lett 228(1):29-32. doi: 10.1016/S0304-3940(97)00358-3
35. Manzanares D, Gonzalez C, Ivonnet P, Chen RS, Valencia-Gattas M, Conner GE, Larsson HP, Salathe M (2011) Functional apical large conductance, Ca²⁺-activated, and voltage-dependent K⁺ channels are required for maintenance of airway surface liquid volume. J Biol Chem 286(22):19830-9. doi: 10.1074/jbc.M110.185074
36. McDonald FJ, Yang B, Hrstka RF, Drummond HA, Tarr DE, McCray PB Jr, Stokes JB, Welsh MJ, Williamson RA (1999) Disruption of the beta subunit of the epithelial Na⁺ channel in mice: hyperkalemia and neonatal death associated with a pseudohypoaldosteronism phenotype. Proc Natl Acad Sci USA 96(4):1727-31. doi: 10.1073/pnas.96.4.1727

37. Nie HG, Chen L, Han DY, Li J, Song WF, Wei SP, Fang XH, Gu X, Matalon S, Ji HL (2009a) Regulation of epithelial sodium channels by cGMP/PKGII. *J Physiol* 587(11):2663-76. doi: 10.1113/jphysiol.2009.170324
38. Nie HG, Tucker T, Su XF, Na T, Peng JB, Smith PR, Idell S, Ji HL (2009b) Expression and regulation of epithelial Na⁺ channels by nucleotides in pleural mesothelial cells. *Am J Respir Cell Mol Biol* 40(5):543-54. doi: 10.1165/rcmb.2008-0166OC
39. Salomon JJ, Muchitsch VE, Gausterer JC, Schwagerus E, Huwer H, Daum N, Lehr CM, Ehrhardt C (2014) The cell line NCI-H441 is a useful in vitro model for transport studies of human distal lung epithelial barrier. *Mol Pharm* 11(3):995-1006. doi: 10.1021/mp4006535
40. Schönberger M, Althaus M, Fronius M, Clauss W, Trauner D (2014) Controlling epithelial sodium channels with light using photoswitchable amilorides. *Nat Chem* 6(8):712-9. doi: 10.1038/nchem.2004
41. Story GM, Peier AM, Reeve AJ, Eid SR, Mosbacher J, Hricik TR, Earley TJ, Hergarden AC, Andersson DA, Hwang SW, McIntyre P, Jegla T, Bevan S, Patapoutian A (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell* 112(6):819-29. doi: 10.1016/S0092-8674(03)00158-2
42. Tarran R (2004) Regulation of airway surface liquid volume and mucus transport by active ion transport. *Proc Am Thorac Soc* 1(1):42-6. **PMID 16113411**
43. Waldmann R, Champigny G, Bassilana F, Voilley N, Lazdunski M (1995) Molecular cloning and functional expression of a novel amiloride-sensitive Na⁺ channel. *J Biol Chem* 270(46):27411-4. doi: 10.1074/jbc.270.46.27411
44. Wesch D, Althaus M, Miranda P, Cruz-Muros I, Fronius M, González-Hernández T, Clauss WG, Alvarez de la Rosa D, Giraldez T (2012) Differential N termini in epithelial Na⁺

- channel δ -subunit isoforms modulate channel trafficking to the membrane. *Am J Physiol Cell Physiol* 302(6):C868-79. doi: 10.1152/ajpcell.00255.2011
45. Yamamura H, Ugawa S, Ueda T, Nagao M, Shimada S (2004a) Protons activate the delta-subunit of the epithelial Na^+ channel in humans. *J Biol Chem* 279(13):12529-34. doi: 10.1074/jbc.M400274200
46. Yamamura H, Ugawa S, Ueda T, Nagao M, Shimada S (2004b) Capsazepine is a novel activator of the delta subunit of the human epithelial Na^+ channel. *J Biol Chem* 279(43):44483-9. doi: 10.1074/jbc.M408929200
47. Yamamura H, Ugawa S, Ueda T, Nagao M, Shimada S (2005a) Icilin activates the delta-subunit of the human epithelial Na^+ channel. *Mol Pharmacol* 68(4):1142-7. doi: 10.1124/mol.104.010850
48. Yamamura H, Ugawa S, Ueda T, Shimada S (2005b) Evans blue is a specific antagonist of the human epithelial Na^+ channel delta-subunit. *J Pharmacol Exp Ther* 315(2):965-9. doi: 10.1124/jpet.105.092775
49. Yamamura H, Ugawa S, Ueda T, Nagao M, Shimada S (2008) Epithelial Na^+ channel delta subunit mediates acid-induced ATP release in the human skin. *Biochem Biophys Res Commun* 373(1):155-8. doi: 10.1016/j.bbrc.2008.06.008
50. Zhao RZ, Nie HG, Su XF, Han DY, Lee A, Huang Y, Chang Y, Matalon S, Ji HL (2012) Characterization of a novel splice variant of δ ENaC subunit in human lungs. *Am J Physiol Lung Cell Mol Physiol* 302(12):L1262-72. doi: 10.1152/ajplung.00331.2011