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RESEARCH ARTICLE

Specific inhibition of epithelial Na^+ channels by antisense oligonucleotides for the treatment of Na^+ hyperabsorption in cystic fibrosis

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Abstract

Background Cystic fibrosis (CF) respiratory epithelia are characterized by a defect Cl^- secretion and an increased Na^+ absorption through epithelial Na^+ channels (ENaC). The present study aimed to find an effective inhibitor of human ENaC with respect to replacing amiloride therapy for CF patients. Therefore, we developed specific antisense oligonucleotides (AON) that efficiently suppress Na^+ hyperabsorption by inhibiting the expression of the α-ENaC subunit.

Methods We heterologously expressed ENaC in oocytes of *Xenopus laevis* for mass screening of AON. Additionally, primary cultures of human nasal epithelia were transfected with AON and were used for Ussing chamber experiments, as well as biochemical and fluorescence optical analyses.

Results Screening of several AON by co-injection or sequential microinjection of AON and ENaC mRNA in *X. laevis* oocytes led to a sustained decrease in amiloride-sensitive current and conductance. Using primary cultures of human nasal epithelia, we show that AON effectively suppress amiloride-sensitive Na^+ absorption mediated by ENaC in CF and non-CF tissues. In western blot experiments, it could be shown that the amount of ENaC protein is effectively reduced after AON transfection.

Conclusions Our data comprise an initial step towards a preclinical test with AON to reduce Na^+ hyperabsorption in CF epithelia. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords antisense oligonucleotides; cystic fibrosis; epithelial Na^+ channel; Na^+ hyperabsorption

Introduction

Na^+ absorption in epithelia lining e.g. the distal nephron, the ducts of secretory glands, the distal colon and the respiratory airways is mainly mediated by the epithelial Na^+ channel in the apical membranes (ENaC) [1]. ENaC mediates the uptake of Na^+ from the luminal side into the cells using a gradient that is maintained by the basolateral Na^+/K^+-ATPase. These channels are characterized by their high affinity to the K^+-sparing diuretic amiloride ($K_{1/2} \sim 0.1$ μM). ENaC exhibits a very low single-channel conductance of approximately 3–5 pS and shows extremely slow gating in the range of seconds [2]. The channel is composed of up to four subunits (α, β, γ and δ) that share 35% homology at the amino acid level [3]. Although the α-subunit alone is able to form a functional low conductance channel, the

β- and γ-subunits accomplish regulatory functions that are necessary for proper channel gating [4]. The δ-ENaC subunit can also form amiloride-sensitive Na^+ channels when co-expressed along with β and γ accessory subunits in heterologous cell systems; however, the generated currents are different from that of the classical subunit composition (α, β and γ) [5]. Furthermore, the δ-subunit shows a different tissue distribution because it is expressed mainly in brain, heart and pancreas and may be activated by external protons [5,6]. Recently, this subunit was also detected in human respiratory epithelium [7]. The channel stoichiometry is still a matter of debate, although it is hypothesized that the functional ENaC exhibits a tetrameric structure of two α-subunits, one β- and one γ-subunit [8]. Other studies suggest a nonameric structure (α_3, β_3 and γ_3) [9,10], although, recently, a trimeric structure was postulated [11]. Defects in ENaC cause diseases such as Liddle's syndrome and pseudohypoaldosteronism [12,13]. Furthermore, ENaC is involved in the pathogenesis of cystic fibrosis (CF), the most frequent genetic disease in the Caucasian population [14,15]. CF is the result of mutations in a gene coding for an epithelial Cl^- channel termed cystic fibrosis transmembrane conductance regulator (CFTR). Beside defective Cl^- secretion, CF epithelia are characterized by a dramatic increase of Na^+ absorption via ENaC [14]. Furthermore, it was shown, in a β-ENaC overexpressing mouse model, that hyperabsorption of Na^+ by ENaC is sufficient to evoke the typical symptoms of cystic fibrosis-like lung disease that is found in CF patients, such as airway surface liquid depletion, mucus retention and neutrophilic inflammation [16]. Previously, we demonstrated that ENaC shows an upregulated subunit expression in CF respiratory epithelia compared to non-CF epithelia [17]. Although the molecular mechanism of ENaC Na^+ hyperabsorption in CF epithelia is not yet fully understood, the ENaC inhibitors amiloride and benzamil were used for inhalation to reduce the Na^+ hyperabsorption [18]. Furthermore, a recent study reported that the amiloride therapy demonstrated therapeutic benefits on airway inflammation in β-ENaC-overexpressing mice when started directly after birth [19]. The positive effects of amiloride and benzamil do not last long enough to yield sustained relief for patients who already suffer from CF-related symptoms [20,21]. Therefore, the present study aimed to develop a method that leads to the inhibition of Na^+ hyperabsorption through ENaC in CF respiratory epithelia. We developed specific antisense oligonucleotides (AON) that resulted in a potent inhibition of ENaC. AON are synthetic DNA molecules with a sequence complementary to a specific mRNA sequence. Binding of the AON complementary to the mRNA transcript evokes a blockage of the translation and stimulates degradation of the mRNA by lysosomes [22]. Previous studies showed that AON directed against the α-subunit of ENaC had the ability to block nonselective cation channels in rat alveolar type II cells [23] and human B lymphocytes [24]. To specifically silence ENaC with AON, we developed a method for the selection of the target sequence on ENaC that allows designing AON effective for related molecules from different tissues. In a first step, we used the *Xenopus laevis* oocyte expression system to test the effectiveness of the AON in silencing ENaC. Measuring the amiloride-sensitive membrane current and conductance mediated by ENaC, we showed that two AON of different sequences prevent the expression of ENaC in oocytes or suppress ENaC effectively when already expressed. In a second step, we used primary cultured human CF and non-CF nasal epithelia cells, which were shown to express ENaC as the dominant cation transport system [14,25]. Because of their accessibility and the close morphologic and cell-physiologic similarity to the lower regions of the respiratory tract, primary cultures of human airway epithelium have proven to be very useful for studying ion transport in these epithelia [26]. Because there is no available animal model for ENaC hyperabsorption, such primary cultured cells offer a useful model for demonstrating ion transport defects in CF. To test the inhibitory effect, we transfected the cells with AON after measuring transepithelial amiloride-sensitive currents mediated by ENaC. Statistical analyses revealed that the AON suppressed more than 66% of Na^+ absorption via ENaC. From these data we conclude that ENaC-specific AON could circumvent the shortcomings of the conventional amiloride inhalation therapy in cystic fibrosis. In addition to the first and second generation of ENaC suppressors (amiloride, benzamil and phenamil [27]), the AON molecules could form a novel generation of ENaC inhibiting substances.

Material and Methods

AON screening using the oocyte expression system

Oocyte preparation

Oocyte extraction and technical procedures are described in more detail elsewhere [28]. Briefly, *X. laevis* females were purchased from African *Xenopus* Facility (Knysna, Republic of South Africa). Anaesthetized frogs (tricaine: MS222, 1.5 g/l, pH 7.0) underwent surgery to remove ovarian lobes, and then the incision was sutured and animals were returned to the tank. Oocytes were defolliculated by incubation for 2 h in Oocyte Ringer (Ori; for composition, see below) with 1 mg/ml collagenase (Serva, Heidelberg, Germany) and subsequently washed for 10 min with Ca^{2+}-free Ringer to remove the follicle cell layer. mRNA or other compounds were microinjected in adjustable amounts using a nanoliter pump (Drummond Nanoject, Broomall, PA, USA). The RNA-injected oocytes (2 ng of mRNA for each ENaC subunit) were allowed 1 day to express ENaC in an incubator at 16 °C. During this time, the oocytes were incubated in an ENaC storage Ringer (ESR; for composition, see below). Afterwards, 23 ng of the respective AON were injected per oocyte.

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Only healthy-looking, full-grown oocytes (stages V–VI) were used for the experiments that were performed at room temperature (approximately 22 °C).

Two microelectrode voltage clamp

The oocyte was placed in a small (0.3 ml) Plexiglas chamber and continuously perfused at adjustable rates. The clamp circuit consisted of two intracellular microelectrodes (for voltage and current) with resistances of approximately 1 MΩ, and two Ag/AgCl pellets in the bath to provide the reference and the virtual ground circuitry. The clamp amplifier (OC-725C; Warner Instruments, Hamden, CT, USA) was interfaced via a series of anti-aliasing filters and digital control circuits with two acquisition boards (Model 310B; Dalanco Spry, Rochester, NY, USA) equipped each with a digital signal processor (DSP), four channels for high-speed 14-bit A/D conversion and two-high speed, 12-bit D/A converters. The DSP boards were programmed to generate the clamp command and to digitize and preprocess the raw data. They were linked to the host computer for final data processing, display, storage and user interface. Our oocyte setup allowed simultaneous and continuous monitoring of the membrane current (I_m), conductance (G_m) and capacitance (C_m). C_m was recorded during each experiment at five different frequencies and is considered as a measure of the membrane surface area. The amiloride-sensitive ENaC current (I_{Na}) and conductance (G_{Na}) were evaluated as the difference before and after application of 50 μM amiloride. By convention, a current carried by positive charges moving from the bath into the cell is termed 'inward', considered negative and plotted downwards in all graphs.

AON design

We designed two AON with sequences complementary to the mRNA of both rat and human α-ENaC subunit. The free Vienna RNA software package (http://www.tbi.univie.ac.at/~ivo/RNA/) was used to compute the predicted secondary structures [29] of rat and human α-ENaC mRNA at 31 different temperatures in the range of 4–36 °C (accession numbers: α-rat ENaC: X70497, α-human EnaC: NM_001038). Using custom made software, each base was assigned a score based on the probability of it being in a single-stranded conformation. We then used further software to search for sequences of 16 bases that are common to the two species and have the highest combined single-strand score, with the rationale being that single-stranded RNA is energetically more favorable to AON binding. The two AON that we termed 'green AON' (gAON; 5′-TGG ATG GTG GTG CTG T-3′) and 'black AON' (bAON; 5′-TTG AAG AAG ATG TTG A-3′), correspond to α-human ENaC mRNA at positions 290 and 1670, respectively, and to α-rat ENaC mRNA at positions 347 and 1733. The actual molecules were synthesized as 2′-*O*-methyl-RNA phosphorothioates by Eurogentec S.A. (Liège, Belgium). This renders the molecules more resistant to nuclease attack and blends the properties of both DNA and RNA. Because RNA has a greater affinity for mRNA than does DNA, this enables the molecule to form a tighter bond with its target.

Solutions

The composition of the basic ORi was (in mM): 90 NaCl, 3 KCl, 2 $CaCl_2$ and 5 HEPES (*N*-2-hydroxylethylpiperazine-*N*-ethanesulfonic acid). The low Na^+ solution (LNS) differed from ORi by having only 15 NaCl and an additional 75 *N*-methyl-D-glucamine (NMDG) chloride. The ESR contained 10 NaCl and 80 NMDG chloride, in addition to the other compounds (same as ORi or LNS). The pH of all Ringer solutions was adjusted to 7.6 with NaOH or HCl, accordingly. Unless otherwise stated, substances were purchased from Roth (Karlsruhe, Germany).

AON testing in human nasal epithelium (HNE)

Patients

We obtained nasal specimens from CF and non-CF patients undergoing nasal surgery. The specimens were typically nasal polyps or nasal turbinates of patients suffering from chronic sinusitis. The study was approved by the committees for human studies of the University of Muenster (Ethik Kommission Muenster) and the University of Giessen (Ethik Kommission der Justus-Liebig-Universitaet Giessen). Declaration of Helsinki protocols were followed and all patients (or their parents, respectively) provided their written, informed consent.

Cell culture

Primary cell culture of HNE was performed as described before [30]. Briefly, the CF and non-CF nasal epithelial cells were isolated by enzymatic digestion for 24–48 h and subsequently seeded on permeable collagen filters with a diameter of 14 mm (Cellagen TM dics CD 24; ICN Biomedicals, Costa Mesa, CA, USA) for Ussing chamber experiments. For biochemical or fluorescence optical analyses, the cells were seeded on collagen coated (0.15 mg/ml, collagen type I; Sigma, Deisenhofen, Germany) cell culture dishes or collagen coated glass cover slips, respectively. The cells were cultured with serum-free F-12 nutrient mixture (Ham) (Invitrogen/Gibco, Karlsruhe, Germany) supplemented with the following agents: insulin (2 μg/ml) (Invitrogen/Gibco), epidermal growth factor (13 ng/ml) (Sigma), endothelial cell growth supplement (7.5 μg/ml) (Becton Dickinson GmbH, Heidelberg, Germany), triiodo-thyronine (3 nM) (Sigma), hydrocortisone (100 nM) (Sigma), gentamycin (10 μg/ml) (Biochrom AG, Berlin, Germany), penicillin/streptomycin (100 U/ml) (Invitrogen/Gibco), L-glutamin (2 mM) (Invitrogen/Gibco) and transferrin (4 μg/ml) (Invitrogen/Gibco). Previously, it was shown that these supplements had no effect on the electrical parameters of the HNE [31]. Cells were incubated in 95% air and 5% CO_2 at 37 °C.

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Transepithelial measurements

After reaching confluence (7–9 days after seeding), the nasal epithelial cells were mounted in modified Ussing chambers. The two compartments of the chamber were continuously perfused with cell culture Ringer at 37 °C. Voltage and current electrodes (AgCl wires) were used and were electrically connected to the chamber by KCl-agar bridges. The current electrodes held the transepithelial potential (V_t), which is established by an active transport of ions to zero. The required current of compensation, termed the short circuit current (I_{sc}), depends on changes in conductance of the epithelium, which is generated by perfusion of different solutions. Na^+ absorption through ENaC was assessed as short-circuit current (I_{SC}) and conductance (G_t) in presence and absence of amiloride (100 μM). To determine the overall Na^+ absorption of the cells, we removed Na^+ from the apical Ringer in a second step. I_{SC} was continuously monitored using the computer software ImpDsp1.4 (Professor Willy Van Driessche, KU Leuven, Leuven, Belgium). The measured area had a size of 0.5 cm^2, whereas the electrical parameters were normalized to an area of 1 cm^2.

Transfection procedures

The 16-bp (5′-TGG ATG GTG GTG CTG T-3′) antisense-nucleotides, the respective sense control (sense-AON, 5′-ACA ACA CCA CCA TCC A-3′) and the fluorescein coupled AON (5′ Fam-AON) were synthesized with phosphorothioate backbone modifications commercially by Metabion (Martinsried, Germany). Cells of human nasal epithelia were measured after reaching confluence in modified Ussing chambers and the bioelectrical parameters were recorded. Subsequently, the cells were transfected with AON (0.15 μg of AON per 14 mm collagen filter) following the standard protocol of the Effectene transfection reagent (Qiagen, Hilden, Germany). Afterwards, the cells were incubated for 36 h and the functional properties were measured again. For the biochemical and fluorescence optical analyses, the cells were transfected in the same way as described before but on the special cell culture support, respectively, and analysed after 36 h of incubation.

Solutions

Cell culture Ringer comprised: 130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES. In the used sodium free solution, NaCl was replaced by an equivalent amount of tetramethylammonium chloride.

Protein biochemistry

For protein isolation, cells were washed twice with phosphate-buffered saline (PBS) on ice and scraped off with ice-cold lysis buffer (1 mM Tris, 15 mM NaCl, 0.2 mM ethylenediaminetetraacetic acid, 2% Triton X-100). Protease inhibitor cocktail was added to this detergent mixture (10 mM leupeptin, 1 mg/ml trypsininhibitor, 25 μM Pefablock, 100 mM phenylmethanesulfonyl fluoride). The cell lysate was then homogenized by repetitive passing through a sterile syringe (diameter 0.9 mm; Braun, Melsungen, Germany). Afterwards, the homogenized lysate was centrifuged at 4 °C at 4000 ***g*** for 30 min, and the supernatant was transferred into a new collection tube. The concentration of the proteins in the supernatant was measured using the Bradford test [32]. For the detection of the α-ENaC subunit, 30 μg of total membrane proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% acrylamide) and transferred to a polyvinylidenfluoride (PVDF) membrane. Nonspecific binding sites were blocked for 2 h by 5% nonfat dry milk in Tris-buffered saline/Tween (TBST) (10 mM Tris HCl, pH 7.4; 140 mM NaCl; 0.3% Tween 20). The ENaC α-subunit was detected with a rabbit anti-α-ENaC antibody (Dianova, Hamburg, Germany) at a concentration of 1 : 2500 diluted in 5% nonfat dry milk/TBST at 4 °C overnight. After washing in TBST, the membrane was incubated for 1 h at room temperature with goat anti-rabbit Ig Gs (immunoglobulin G) conjugated with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1 : 10 000 in 5% nonfat dry milk/TBST. The membrane was washed again in TBST and detection was carried out with NBT (nitroblue tetrazolium) and 5-bromo-4-chloro-3- indolyl phosphate.

To determine the protein amounts of the bands detected by the specific anti α-ENaC antibody in western blot experiments, we digitised the PVDF membranes and analysed the blot semi-quantitatively by densitometry using ImageJ analysis software 1.36 [33].

Fluorescence optical analyses

For detection of the cellular uptake of the AON and their distribution, nasal epithelial cells were seeded on glass cover slips and transfected with fluorescein-labeled AON (5′ Fam-AON, absorption peak by 492 nm). Therefore, cells were gently washed five times in PBS (in mM: 140 NaCl, 2 KCl, 4 Na_2HPO_4, 1 KH_2PO_4, pH 7.4) and cell fixation was performed with 0.05% glutaraldehyde for 10 min at 37 °C. Subsequently, the cells were washed again in PBS and once in water to remove salt, and were then mounted in Moviol/Dabco (Roth, Karlsruhe, Germany). Images were acquired by using the Axio Vision software, version 4.6, with an inverted fluorescence microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany) equipped with a × 100 1.45 oil immersion objective and fluorescence filter.

Statistical analysis

Where applicable, data are expressed as the arithmetic mean ± SEM; n is the number of oocytes and N is the number of female donors. In the case of experiments with primary cultured monolayers, n is the number of patients. Statistical analysis was performed using the t-test where appropriate, and a significant difference was assumed at $^*p < 0.01$ or $^{**}p < 0.001$.

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Results

Basic parameters of ENaC expressing oocytes

When using the *X. laevis* oocyte as a heterologous expression system for transport proteins, it is absolutely mandatory to make sure that the foreign transporter does not significantly influence or even profoundly alter intraoocyte concentrations of ions and other relevant substances. In the case of ENaC, injection of mRNA coding for α-β-γ-ENaC leads to expression of a huge number of functional Na^+ channels, amounting up to 10^9 active copies of ENaC per oocyte [34]. This large number of channels would severely change intraoocyte Na^+ concentration ($[Na^+]_i$), thereby drastically altering ENaC gating and conducting properties, subsequently leading to ENaC inactivation. To circumvent these problems, we stored micro-injected oocytes immediately in low Na^+ solutions and kept them under low Na^+ conditions until the electrophysiological experiments (i.e. 10 and 15 mM, respectively). Furthermore, during the experiments, we prevented intracellular accumulation of Na^+ via the expressed ENaC by clamping the current to zero (the software-driven current clamp mode; see Materials and methods). Then we only briefly clamped to −60 mV to perform noise analysis and to assess the amiloride-sensitive current. α-β-γ-ENaC mRNA-injected oocytes expressed ENaC within 20 h and could be used for at least the next 5 days. The extracellular Na^+ concentrations used in the present study are close to the average intracellular Na^+ concentration of the oocytes, as reported in other studies [35]. As already stated, control experiments revealed that H_2O-injected oocytes and non-injected oocytes showed no differences in membrane voltage (V_m), current (I_m), conductance (G_m) and capacitance (C_m). Therefore, the term 'controls' refers to non-injected oocytes. The initial electrophysiological parameters of ENaC expressing oocytes and controls are summarized in Table 1.

Table 1. Initial electrophysiological parameters of ENaC expressing oocytes and controls

	Parameter	Initial value	*N*	*n*
V_m	ENaC	6.3 ± 1.5 mV	13	100
	Controls	-47 ± 2 mV	13	59
G_m	ENaC	113.5 ± 10.6 µS	13	100
	Controls	~ 1 µS	13	59
I_{Na}	ENaC	3.8 ± 0.3 µA	14	117
	Controls	–	–	–
C_m	ENaC	164 ± 3 nF	13	100
	Controls	158 ± 6 nF	13	59

The results are presented as the mean ± SEM; *n* is the number of oocytes and *N* is the number of female donors; membrane voltage (V_m), conductance (G_m), amiloride-sensitive current (I_{Na}), capacitance (C_m).

Development and test for specific effects of AON on ENaC expression in X. laevis oocytes

We designed several AON and tested them for efficacy on ENaC expressed in *X. laevis* oocytes. Two AON, gAON and bAON, proved to be highly specific in ENaC down-regulation. To achieve strict control on the AON amount delivered in the oocyte cytoplasm, all AON experiments were performed using the microinjection technique as a delivery method [36]. We pursued two lines of investigation aiming to assess the effects of gAON and bAON on ENaC expression: the 'simultaneous' approach, in which the AON was injected together with the ENaC mRNA mixture, and the 'sequential' approach, in which oocytes were first allowed to express ENaC before the AON microinjection step.

The simultaneous AON approach. Coinjection of gAON with α-β-γ-ENaC successfully inhibits channel expression, as demonstrated by the negative V_m and the virtual lack of amiloride sensitivity of the I_m and G_m in the presence of gAON (Figure 1). In this set of experiments, oocytes injected with ENaC alone exhibited a I_m of 6.4 ± 1.0 µA and G_m of 217 ± 30 µS, whereas coinjection of gAON reduced these values to 0.4 ± 0.2 µA and 8 ± 5 µS,

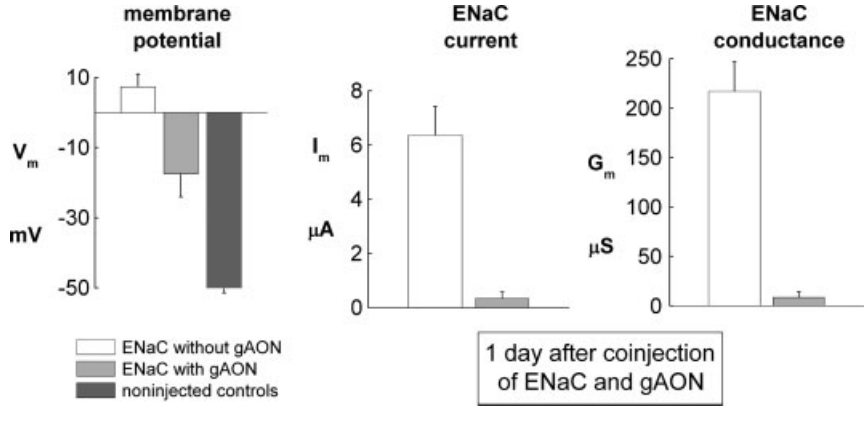

Figure 1. Blockage of ENaC expression by simultaneous injection of ENaC mRNA and gAON. The negative resting membrane potential (V_m) and the very low amiloride-sensitive current (I_m) and conductance (G_m) indicate successful inhibition of ENaC expression by the antisense oligonucleotides. Shown are means ($n = 19$, $N = 3$; where *n* is the number of oocytes and *N* is the number of female donors)

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respectively. The results for bAON are not shown because they agree in every aspect with those given for gAON.

The sequential AON approach. Sequential microinjection of ENaC mRNA followed 24 h later by AON led to an exponential-like decrease in amiloride-sensitive current (I_{Na}) and conductance (G_{Na}), with highly variable time constants ranging from 2 h to 2 days, accompanied by a gradual shift of the membrane potential from positive to negative values (data not shown). Although bAON (Figure 2B) appeared to be more efficient and almost completely inhibited ENaC activity after 1 day, gAON

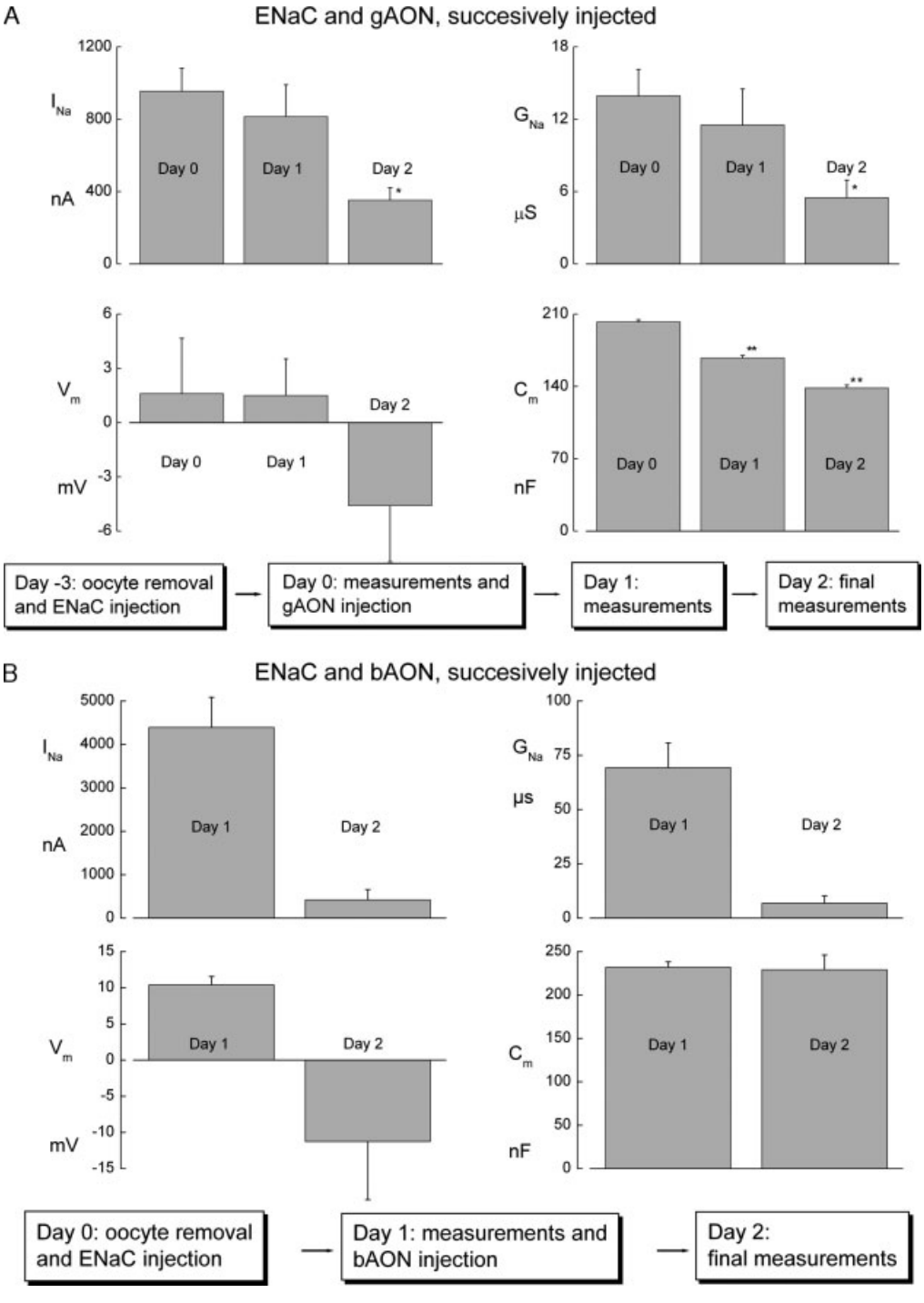

Figure 2. Blockage of ENaC expression by sequential injection of ENaC mRNA followed by gAON or bAON. Oocytes were allowed to express ENaC for 2 days, then the ENaC activity (I_{Na}, G_{Na}) was measured and the AON injected (day 0). ENaC activity was again measured on subsequent days (day 1, day 2) and was found to decrease in parallel with a recovery of the membrane resting potential (V_m) towards negative values. (A) Results for gAON ($n = 10$, $N = 2$): inhibition was slow, with significant changes in parameters only at day 2. Membrane capacitance (C_m) exhibited a significant, time-dependent decrease. (B) Results for bAON ($n = 8$, $N = 2$): ENaC activity was almost completely lost after 1 day, without effects on the membrane surface area (C_m remains constant)

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(Figure 2A) took 48 h to reverse the membrane potential and to lower I_{Na} and G_{Na} significantly. On the other hand, ENaC-expressing oocytes injected with gAON exhibited a significant and time-dependent decrease of membrane surface area measured as C_m. Because it was not observed in the absence of ENaC expression, this effect also appears to be specific for the AON interaction with the ENaC mRNA. However, the data are insufficient for further speculation, and we cannot assess whether the decrease in C_m is caused by endocytotic removal of ENaC from the plasma membrane, or some other imbalance in the flow of intracellular membranes.

Inhibition of Na^+ absorption in HNE

To analyse the inhibitory AON effect on the functional level, we used confluent monolayers of primary cultured human CF and non-CF nasal epithelia in Ussing chamber measurements. In experiments using non-CF nasal epithelial cells, I_{SC} is markedly inhibited by amiloride, demonstrating a substantial Na^+ absorption via ENaC (Figure 3A, upper trace). In a second step, we removed Na^+ from the apical Ringer to determine the overall Na^+ absorption of HNE. Subsequently, the same monolayer was transfected with gAON and measured again 36 h later. Transfection of HNE monolayers resulted in drastically reduced response of the monolayers to amiloride (Figure 3A, lower trace), indicating that the expression of ENaC is significantly reduced by the gAON. The statistical evaluation of the performed measurements revealed that the transfection of gAON reduces the amiloride-sensitive Na^+ absorption in non-CF HNE by approximately 66% (Figure 3B). Measurements of CF monolayers with marked Na^+ hyperabsorption also revealed a distinct reduction of the amiloride-sensitive I_{SC} after AON transfection. The measurement of a CF monolayer (Figure 4A) showed an elevated ENaC current of 79.3% in relation to the total Na^+ absorption (upper trace). After AON transfection, the amiloride-sensitive ENaC was significantly reduced by approximately 80% (Figure 4A, lower trace). The statistical evaluation of the performed CF measurements revealed that the AON transfection reduces the amiloride-sensitive Na^+ absorption by approximately 75% (Figure 4B). For control experiments, monolayers were transfected with sense-oligonucleotides (sense-AON) and in another set of experiments with an equivalent amount of H_2O instead of AON. These mock transfections showed no effects on the amiloride-sensitive Na^+ absorption (data not shown).

To localize the AON distribution and uptake patterns in single cells, fluorescein-labeled AON (5′ Fam-AON) were used for transfection of non-CF epithelial cells on glass cover slips and analysed by fluorescent microscopy. With this method, we were able to show, that the labeled AON successfully enter primary epithelial cells after transfection and that they are existent in an adequate amount to evoke reduction of ENaC expression (Figure 5).

To analyse the inhibitory effect of AON treatment on the protein level, we carried out western blot analyses with a specific anti α-ENaC antibody. We found a marked suppression of the α-ENaC protein in AON transfected human non-CF nasal epithelial cells compared to nontransfected cells (Figure 6). Using statistic evaluation, we could show that the expression of the α-ENaC protein was reduced by approximately 47% in the AON transfected cells.

Discussion

Besides the defective Cl^- secretion mediated by the mutated CFTR protein, the drastically increased Na^+

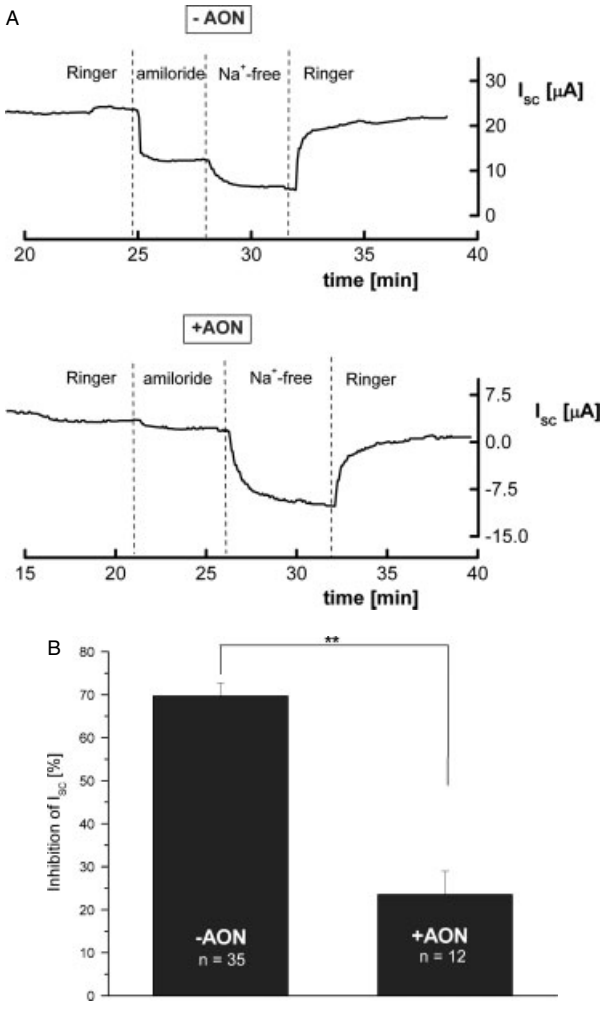

Figure 3. Effect of gAON treatment in human non-CF nasal epithelia cells on transepithelial ENaC current. (A) Time course of a typical experiment. Shown is the short-circuit current (I_{SC}) of cultured monolayers from human non-CF nasal epithelia. I_{SC} is markedly inhibited by amiloride (100 µM), demonstrating the Na^+ absorption via ENaC. In a second step, we removed Na^+ from the apical Ringer to determine the overall Na^+ absorption of the cells. The upper trace (before gAON transfection) shows a large amiloride response. After gAON transfection, this large response is markedly reduced (lower trace). (B) Summary of measured epithelia. Statistical evaluation of the I_{SC} measured before and after gAON transfection of cultured human non-CF epithelial cells. The amiloride-sensitive ENaC current is reduced by 66.2% (− AON, $n = 35$; + AON, $n = 12$)

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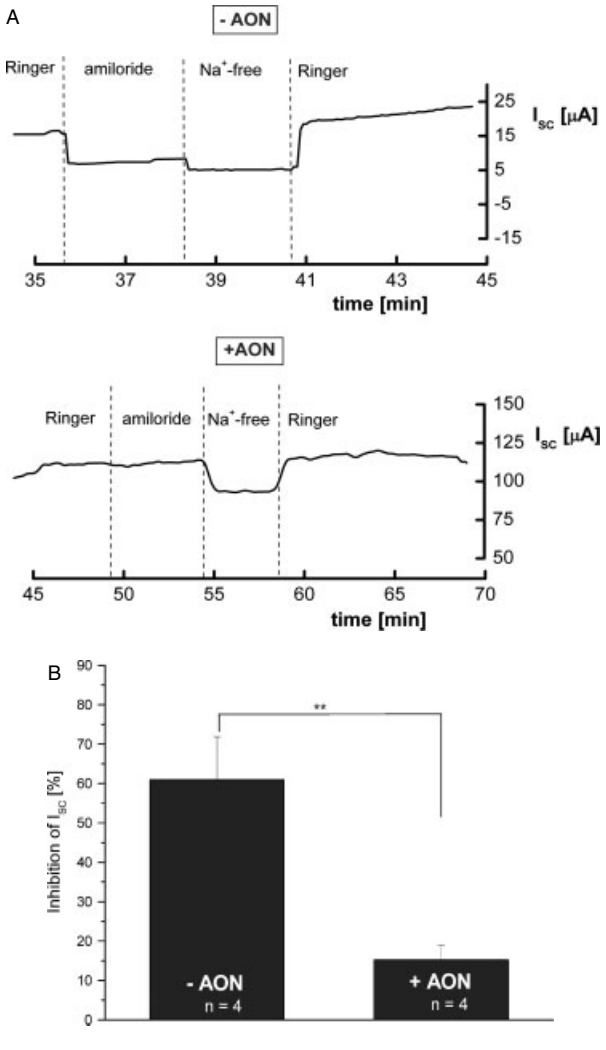

Figure 4. Effect of gAON treatment in human CF nasal epithelia cells on transepithelial ENaC current. (A) Time course of a typical experiment. Shown is the I_{SC} of cultured monolayers from human CF nasal epithelia. I_{SC} is markedly inhibited by amiloride (100 μM), demonstrating the Na^+ absorption via ENaC. In a second step, we removed Na^+ from the apical Ringer to determine overall Na^+ absorption of the cells. The upper trace (before gAON transfection) shows a large amiloride response. After gAON transfection, this large response is markedly reduced (lower trace). (B) Summary of measured epithelia. Statistical evaluation of the I_{SC} measured before and after gAON transfection of cultured CF epithelial cells. The amiloride-sensitive ENaC current is reduced by 75.1% (− AON, $n = 4$; + AON, $n = 4$)

absorption through ENaC is one of the most obvious defects in CF respiratory epithelia. For several years, it has been known that the ENaC activity of cultured CF cells derived from human airways is increased by two- to three-fold compared to non-CF cells [37]. In transepithelial measurements using primary cultures of human respiratory epithelium, these findings regarding CF Na^+ hyperabsorption were confirmed and extended [14,17,30]. Moreover, in an approach using a mouse model with airway-specific overexpression of β-ENaC and intact CFTR function, it could be demonstrated that Na^+ hyperabsorption alone is sufficient to initiate cystic fibrosis-like lung disease [16]. The mechanistic links between altered ion transport in the airway epithelia and regulation of CF Na^+ hyperabsorption are far from being understood. However, there is some evidence that human respiratory ENaC exhibits some functional and structural differences compared to ENaC from other tissues such as the intestine and kidney [25]. Furthermore, the regulation of ENaC is proven to be highly tissue-specific and it is known that local regulators responding to signals near to the channel play an important role in the particular tissue [38].

Suppression of the Na^+ hyperabsorption in CF patients, as demonstrated by the inhalation of high doses of amiloride or analogues such as benzamil, leads to the correction of mucous viscosity by increasing its water content, thereby resulting in improvement of the patient's state of health [21,30]. Because amiloride therapy gives only temporary relief and benzamil is not yet approved, we propose to inhibit the increased Na^+ absorption using AON technology. In the present study, we show basic evidence for the effectiveness of AON, so that the drawbacks of amiloride inhalation could be circumvented by the more persistent reduction of the ENaC expression.

Using the *X. laevis* oocyte expression system, we could clearly demonstrate that the applied AON successfully inhibits ENaC expression in oocytes, as demonstrated by the negative V_m and the lack of amiloride sensitivity of I_m and G_m. With the 'sequential AON approach' (see Results), we show an exponential-like decay in ENaC activity by AON application, with highly variable time constants ranging from 2 h to 2 days. Assuming that the *de novo* synthesis of ENaC is effectively blocked, as the results from the 'simultaneous approach' would suggest, then the residual amiloride-sensitive currents can be explained only by channel molecules that were already synthesized before AON delivery. Furthermore, if no channel insertion into the plasma membrane takes place after AON delivery, then the time constant of the decay of ENaC activity should be more or less constant and proportional to the half-life time of ENaC in the membrane, which is not the case. Although more data are required to confirm this hypothesis, we conclude that the variability in the time course of the inhibition is a result of the existence of highly variable intracellular stores of preformed ENaC molecules.

Several AON were designed and we used ENaC expressing oocytes to identify the most effective AON and rule out the less effective ones. This system allowed AON testing within some days without the need of elaborate and expansive cell culture techniques. Two highly efficient AON (gAON and bAON) were identified and used for all additional experiments.

To go one step further towards *in vivo* application, we used primary cultures of HNE to analyse the inhibitory AON effect. Our data show a strong decrease of ENaC expression similar to the AON effect observed in the oocyte expression system. With Ussing chamber measurements, we could show that the AON application led to a reduction of the amiloride-sensitive ENaC current in human non-CF and in CF nasal epithelial cells. AON inhibited the

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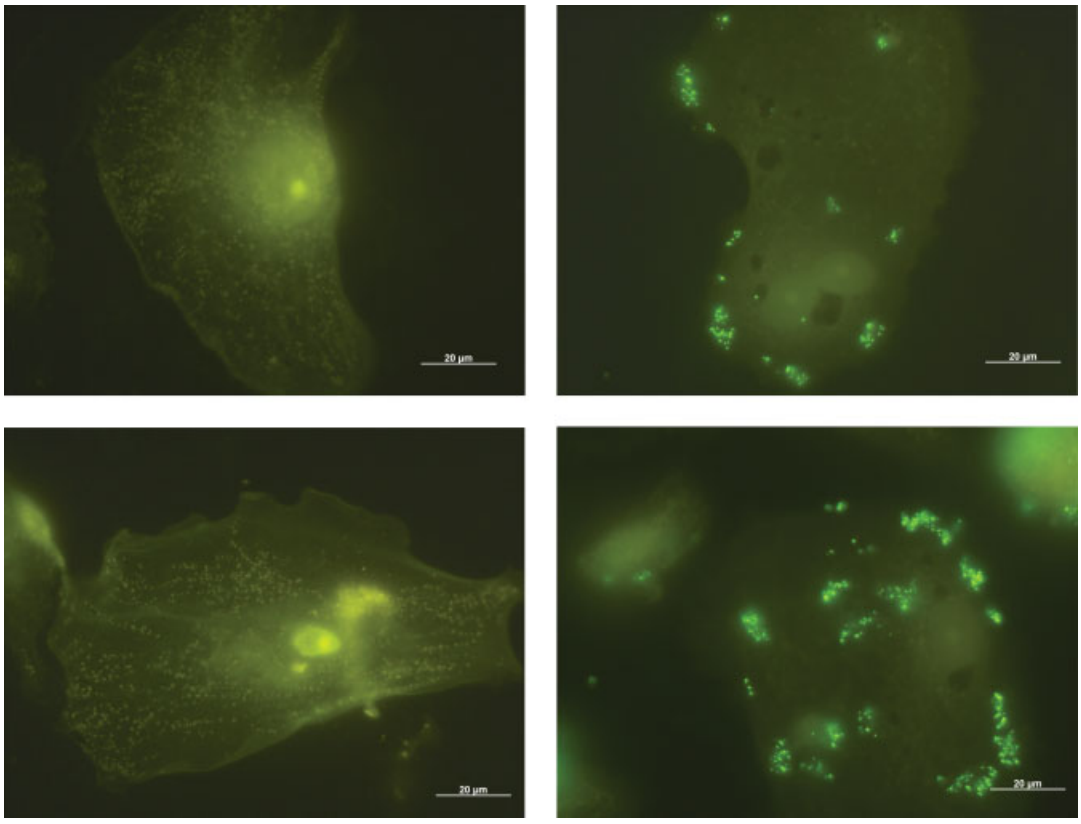

Figure 5. Optical detection of fluorescein-labeled gAON. We detected the fluorescein-labeled gAON 36 h after transfection of the cells. The background staining of the cells is a result of fixation with glutaraldehyde (0.05%), which allows the identification of cell borders. Representative images of this experiment are shown. Images on the right (+ AON) show that the AON successfully enter the epithelial cell after transfection and appear in a sufficient amount in the cells. For comparison, images on the the left (− AON) show untransfected cells (scale = 20 µm)

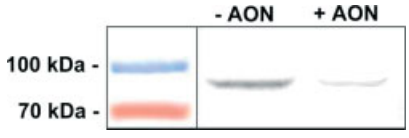

Figure 6. Western blot analyses of gAON transfected and untransfected human non-CF epithelial cells ($n = 6$). Total membrane proteins from human nasal non-CF tissue were isolated using 2% Triton X-100 and separated on a 7.5% SDS-PAGE. To identify ENaC, we used a specific anti-ENaC antibody against this subunit. We detected one specific band of α-ENaC in the range of 100 kDa in the untransfected control and the transfected cells, respectively. The representative image shows that the α-ENaC protein amount is reduced in the gAON transfected cells (+ AON) compared to untransfected cells (− AON)

ENaC current in non-CF nasal cells by approximately 66% and in CF epithelia by approximately 75%, respectively. Furthermore, western blot analyses reveal, on the protein level, that AON decrease ENaC expression very efficiently by approximately 46% in non-CF cells. These data clearly demonstrate that AON are able to specifically inhibit ENaC expression in human CF and non-CF nasal tissue. Therefore, these data are in good accordance with a previous study where AON were targeted against the ENaC α-, β- and γ-subunits in rat alveolar type II cells [23]. Using patch clamp measurements, the authors demonstrated that the application of AON directed against the α-ENaC subunit reduced the density of channels in the plasma membrane. The inhibition by AON directed against the other two (β and γ) subunits did not cause any changes in the channel density and the authors concluded that the α-ENaC subunit is the major conducting part of lung alveolar epithelial cation channels. Therefore, the observations of Jain *et al*. [23] emphasize the findings of the present study, indicating that AON against the α-ENaC subunit comprise a suitable technique for the effective reduction of ENaC expression in the membrane. Moreover, because α-ENaC expression is increased in CF airways compared to non-CF airways [17], targeting this overexpression appears to be a promising way to correct Na^+ hyperabsorption. Estimations showed that AON of 15–17 residues in length would be unique in the entire human genome. Thus, a short AON could be used for the specific blockage of the expression of the target gene without affecting the expression of other genes [39]. To maximize efficacy and safety as well as minimize toxic side-effects, the sequence of the respective AON must be sufficiently specific to silence the target gene. Therefore, we used the Vienna software package as a guide that predicts secondary structure, sequence accessibility and the efficiency and strength of binding, among other factors. Studies show that the AON effect highly depends on the specificity of the target

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sequence and on the sufficient intracellular concentration [40,41]. Although AON are taken up into cells by receptor mediated endocytosis or pinocytosis and are transported within cells to the nuclei, the number of cells that can be reached depends on the route of delivery [42,43]. In turn, this imposes certain limitations on the *in vivo* studies. Therefore, special vehicles such as cationic lipids or liposomes, which are released into the cell after a fusion with the membrane, have proved to be suitable [44]. For that reason, in the present study, we used a lipid based transfection procedure for the experiments with primary human nasal epithelia cells and could demonstrate with fluorescence optical analyses that the AON are efficiently internalized by the cells and are present in a high intracellular concentration.

Subsequent to the development of the antisense-technology almost 30 years ago [45], these molecules were constantly optimized with respect to their target specificity and stability. A multiplicity of different AON applications were already proven in clinical trials for the therapy of diverse human diseases, such as cancer, virus infections, or immune deficiency [42]. AON therapy has already been successfully established in different studies against viral pathogens such as HIV, cytomegalovirus (CMV) and human pappiloma virus, as well as against tumor growth [22,46]. The first available drug on AON basis is called Vitravene™ (Isis Pharmaceuticals, Carlsbad, CA, USA) and is appointed to the treatment of CMV retinitis attended by AIDS. Furthermore, approximately 20 antisense drugs are in clinical development, so that these drugs may prove valuable for the treatment of a wide range of diseases [47,48]. Moreover, recent improvements of AON technology are more than promising [49]. Another possibility for targeting Na^+ hyperabsorption in the respiratory epithelia could be based on specific short interfering RNA (siRNA) directed against ENaC subunits. However, to the best of our knowledge, there are only a few studies of clinical trials using siRNA to knock out proteins involved in human diseases. Furthermore, some recent studies revealed severe drawbacks of an siRNA approach, such as the potential to cause toxicity and immunogenicity [50]. As already mentioned, the AON used in the present study are highly specific for the α-ENaC subunit and we expect that no other proteins will be targeted. Moreover, it has been shown that human respiratory epithelia possess other amiloride-insensitive Na^+ conductances that prevent Na^+ and water depletion of the cells [14,51].

From data originating from *X. laevis* oocytes and the human respiratory epithelium, we expect the AON effect to last for at least 24 h in humans. Nevertheless, repeated applications will be necessary after this period for a sustained suppression of Na^+ hyperabsorption. With regard to CF, AON directed against ENaC are potentially of interest as a replacement for amiloride therapy in CF because they may extend the duration of the ENaC suppressing effect. Therefore, the next step will be a first preclinical test involving the local administration of AON to nasal epithelia of non-CF and CF patients, where the suppressing AON effect could be assessed using the technique of nasal potential difference measurements. In the meantime, we have initiated a preclinical study using nasal spray to locally administer our AON to the nasal epithelia of volunteers and CF patients. In a second step, recently developed inhalation procedures will be tested to deliver the AON to the lungs of CF patients [52]. From these orientating studies, we expect a longlasting suppression of excessive Na^+ absorption in CF by AON therapy, thereby improving the quality of life for patients.

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References

1. Alvarez de la Rosa D, Canessa CM, Fyfe GK, Zhang P. Structure and regulation of amiloride-sensitive sodium channels. *Annu Rev Physiol* 2000; **62**: 573–594.
2. Kellenberger S, Schild L. Epithelial sodium channel/degenerin family of ion channels: a variety of functions for a shared structure. *Phys Rev* 2002; **82**: 735–767.
3. Rossier BC, Pradervand S, Schild L, Hummler E. Epithelial sodium channel and the control of sodium balance: interaction between genetic and environmental factors. *Annu Rev Physiol* 2002; **64**: 877–897.
4. Canessa CM, Schild L, Buell G, *et al*. Amiloride-sensitive epithelial Na^+ channel is made of three homologous subunits. *Nature* 1994; **367**: 463–467.
5. Waldmann R, Champigny G, Bassilana F, Voilley N, Lazdunski M. Molecular cloning and functional expression of a novel amiloride-sensitive Na^+ channel. *J Biol Chem* 1995; **270**: 27411–27414.
6. Ji H-L, Benos DJ. Degenerin sites mediate proton activation of $\delta\beta\gamma$-epithelial sodium channel. *J Biol Chem* 2004; **279**: 26939–26947.
7. Bangel N, Kusche-Vihrog K, Langhorst H, Weber W-M. Cloning of the epithelial sodium channel δ subunit in human nasal epithelium. *Acta Physiol* 2008; **188**: S107.

8. Firsov D, Gautschi I, Merillat A-M, Rossier BC, Schild L. The heterotetrameric architecture of the epithelial sodium channel (ENaC). *EMBO J* 1998; **17**: 344–352.
9. Staruschenko A, Adams E, Booth RE, Stockand JD. Epithelial Na^+ channel subunit stoichiometry. *Biophys J* 2005; **88**: 3966–3975.
10. Snyder PM, Cheng C, Prince LS, Rogers JC, Welsh MJ. Electropyhsiological and biochemical evidence that DEG/ENaC cation channels are composed of nine subunits. *J Biol Chem* 1998; **273**: 681–684.
11. Stockand JD, Staruschenko A, Pochynyuk O, Booth RE, Silverthorn DU. Insight toward epithelial Na^+ channel mechanism revealed by the acid-sensing ion channel 1 structure. *IUBMB Life* 2008; **60**: 620–628.
12. Bubien JK, Ismailow II, Berdiev BK, *et al*. Liddle's disease: abnormal regulation of amiloride-sensitive Na^+ channels by β-subunit mutation. *Am J Physiol* 1996; **270**: C208–C213.
13. Schild L. The epithelial sodium channel: from molecule to disease. *Rev Physiol Biochem Pharmacol* 2004; **151**: 93–107.
14. Rückes-Nilges C, Weber U, Lindemann H, Münker G, Clauss W, Weber W-M. Minor role of Cl^- secretion in non-cystic fibrosis and cystic fibrosis human nasal epithelium. *Cell Physiol Biochem* 1999; **9**: 1–11.
15. Berdiev BK, Ismailow II. Epithelial sodium channels in cystic fibrosis. *Curr Top Membr* 1999; **47**: 351–380.
16. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. *Nature Med* 2004; **10**: 487–493.
17. Bangel N, Dahlhoff C, Sobczak K, Weber WM, Kusche-Vihrog K. Upregulated expression of ENaC in human CF nasal epithelium. *J Cyst Fibros* 2008; **7**: 197–205.
18. Hofmann T, Stutts MJ, Ziersch A, *et al*. Effects of topically applied benzamil and amiloride on nasal potential difference and sodium absorption in cystic fibrosis respiratory epithelium. *Am J Respir Crit Care Med* 1998; **157**: 1844–1849.
19. Zhou Z, Treis D, Schubert SC, *et al*. Preventive but not late amiloride therapy reduces morbidity and mortality of lung disease in betaENaC-overexpressing mice. *Am J Respir Crit Care Med* 2008; **178**: 1245–1256.
20. Tomkiewicz RP, App EM, Zayas JG, *et al*. Amiloride inhalation therapy in cystic fibrosis. *Am Rev Respir Dis* 1993; **148**: 1002–1007.
21. Hofmann T, Stutts MJ, Ziersch A, *et al*. Effects of topically delivered benzamil and amiloride on nasal potential difference and sodium absorption in cystic fibrosis respiratory epithelium. *Am J Respir Crit Care Med* 1998; **157**: 1844–1849.
22. Phillips MI, Gyurko R. Antisense oligonucleotides: new tools for physiology. *News Physiol Sci* 1997; **12**: 99–105.
23. Jain L, Chen XJ, Malik B, Al-Khalili O, Eaton DC. Antisense oligonucleotides against the alpha-subunit of ENaC decrease lung epithelial cation-channel activity. *Am J Physiol* 1999; **276**: L1046–L1051.
24. Ma H-P, Al-Khalili O, Ramosevac S, *et al*. Steroids and exogenous γ-ENaC subunit modulate cation channels formed by α-ENaC in human B lymphocytes. *J Biol Chem* 2004; **279**: 33206–33212.
25. Rückes C, Blank U, Möller K, *et al*. Amiloride-sensitive Na^+ channels in human nasal epithelium are different from classical epithelial Na^+ channels. *Biochem Biophys Res Com* 1997; **237**: 488–491.
26. Gruenert DC, Finkbeiner WE, Widdicombe JH. Culture and transformation of human airway epithelial cells. *Am J Physiol* 1995; **268**: L347–L360.
27. Hirsh AJ, Sabater JR, Zamurs A, *et al*. Evaluation of second generation amiloride analogs as therapy for cystic fibrosis lung disease. *J Pharmacol Exp Ther* 2004; **311**: 929–938.
28. Weber W-M, Clauss W, Cuppens H, Cassiman JJ, Van Driessche W. Capacitance measurements reveal different pathways for the activation of CFTR. *Pflügers Arch – Eur J Physiol* 1999; **438**: 561–569.
29. Hofacker I, Fontana W, Stadtler PF, Bonhoeffer S, Tacker M, Schuster P. Fast folding and comparison of RNA secondary structures. *Monatshefte für Chemie* 1994; **125**: 167–188.
30. Blank U, Clauss W, Weber W-M. Effects of benzamil in human cystic fibrosis airway epithelium. *Cell Physiol Biochem* 1995; **5**: 385–390.
31. Blank U, Rückes C, Clauss W, *et al*. Cystic fibrosis and non-cystic fibrosis human nasal epithelium show analogous Na^+ absorption and reversible block by phenamil. *Pflügers Arch – Eur J Physiol* 1997; **434**: 19–24.
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248–254.
33. Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. *Biophot Intern* 2004; **11**: 36–42.
34. Segal A, Cucu D, Van Driessche W, Weber W-M. Rat ENaC expressed in *Xenopus laevis* oocytes is activated by cAMP and blocked Ni^{2+}. *FEBS Lett* 2002; **515**: 132–138.
35. Weber W-M. Ion currents of *Xenopus laevis* oocytes: State of the art. *Biochim Biophys Acta* 1999; **1421**: 213–233.
36. Segal A, Awayda MS, Eggermont J, Van Driessche W, Weber W-M. Influence of voltage and extracellular Na^+ concentration on amiloride block and transport kinetics of rat ENaC expressed in *Xenopus* oocytes. *Pflügers Arch – Eur J Physiol* 2002; **443**: 882–891.
37. Boucher RC, Stutts MJ, Knowles MR, Cantley L, Gatzy JT. Na^+ transport in cystic fibrosis respiratory epithelia. *J Clin Invest* 1986; **78**: 1245–1252.
38. McNicholas CM, Canessa CM. Diversity of channels generated by different combinations of epithelial channel subunits. *J Gen Physiol* 1997; **109**: 681–692.
39. Stein CA. Exploiting the potential of antisense: beyond phosphorothioate oligodeoxynucleotides. *Chem Biol* 1996; **3**: 319–323.
40. Ho SP, Britton DHO, Stone BA, *et al*. Potent antisense oligonucleotides to the human multidrug resistance-1 mRNA are rationally selected by mapping RNA-accessible sites with oligonucleotide libraries. *Nucleic Acid Res* 1996; **24**: 1901–1907.
41. Kronenwett R, Steidl U, Kirsch M, Sczakiel G, Haas R. Oligodeoxyribonucleotide uptake in primary human hematopoietic cells is enhanced by cationic lipids and depends on the hematopoietic cell subset. *Blood* 1998; **91**: 852–862.
42. Akhtar S, Hughes MD, Khan A, *et al*. The delivery of antisense therapeutics. *Adv Drug Deliv Rev* 2000; **44**: 3–21.
43. Yakubov LA, Deeva EA, Zarytova VF, *et al*. Mechanism of oligonucleotide uptake by cells: involvement of specific receptors? *Proc Natl Acad Sci USA* 1989; **86**: 6454–6458.
44. Wagner RW. Gene inhibition using antisense oligodeoxynucleotides. *Nature* 1994; **372**: 333–335.
45. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci USA* 1978; **75**: 280–284.
46. Wagner RW, Matteucci MD, Grant D, Huang T, Froehler BC. Potent and selective inhibition of gene expression by an antisense heptanucleotide. *Nat Biotechnol* 1996; **14**: 840–844.
47. Akhtar S. Antisense technology: selection and delivery of optimally acting antisense oligonucleotides. *J Drug Target* 1998; **5**: 225–234.
48. Crooke ST. Antisense strategies. *Curr Mol Med* 2004; **4**: 465–487.
49. Yokota T, Takeda S, Lu QL, Partridge TA, Nakamura A, Hoffman EP. A renaissance for antisense oligonucleotide drugs in neurology: exon skipping breaks new ground. *Arch Neurol* 2009; **66**: 32–38.
50. Castanotto D, Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. *Nature* 2009; **457**: 426–433.
51. O'Brodovich H, Yang P, Gandhi S, Otulakowski G. Amiloride-insensitive Na^+ and fluid absorption in the mammalian distal lung. *Am J Physiol Lung Cell Mol Physiol* 2008; **294**: L401–L408.
52. Elfinger M, Geiger J, Hasenpusch G, *et al*. Targeting of the beta(2)-adrenoceptor increases nonviral gene delivery to pulmonary epithelial cells in vitro and lungs in vivo. *J Control Release* 2009; **135**: 234–241.