Correction of Impaired Anion Secretion In CFTR-related Chronic Pulmonary Diseases

Ph.D. thesis

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1. Introduction

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a protein that is mainly localized on the apical surface of polarized epithelial cells in the body. Anion channel function of CFTR is crucial in maintaining the osmotic balance and viscosity of the mucus in the airways, pancreas, intestine, salivary glands, liver, skin, reproductive organs. Composition of mucus in these organs is also altered by CFTR through its effects on other ion transporters, such as regulation of epithelial sodium channel (ENaC) and cooperativity with Cl^-/HCO_3^- transport. The latter plays an important role in the pH homeostasis of mucus, mainly in the airway surface liquid (ASL). Effective mucociliary clearance (MCC) depends on the thickness and composition of ASL and proper ciliary beat frequency. Cystic fibrosis (CF) is a chronic lung disease which is caused by mutations in CFTR gene. In CF the morbidity and mortality are caused by airway destruction, characterized by a vicious cycle of obstruction, inflammation and infection leading to respiratory failure and death. Chronic obstructive pulmonary disease (COPD) is a progressive respiratory disorder consisting of chronic bronchitis and/or emphysema and it is the third leading cause of death worldwide. Compromised mucociliary clearance is the central feature of respiratory pathology in COPD. There is recent evidence from several studies that CFTR activity is reduced in COPD. Improvement of mucus clearance in CF and COPD is key to preventing declines in lung function. Even in CF where CFTR-corrective therapies are available, there is a group of patients (e.g., with nonsense mutations) who will still rely on symptomatic treatment via CFTR-independent approaches. Direct modulation of mucus properties via ion channel targeting could be an effective strategy not only in patients with CF, but also in patients with COPD, especially in those individuals who

suffer from chronic bronchitis and/or mucus stasis and accumulation.

2. Objectives

1. One of the therapeutic approaches for defective CFTR function is activation of alternative anion channels, such as the Ca-activated chloride channel in the airway epithelium. The calcium homeostasis of epithelial cells is regulated by calcium influx from extracellular space, calcium release by intracellular stores and calcium elimination. Our aim was to test the effect of extracellular pH, ATP and divalent cations (including zinc) on the intracellular calcium level of airway epithelial cells to determine the ideal composition of aerosols in which a drug can be applied to improve mucociliary clearance and ASL hydration in diseases with CFTR dysfunction.

2. Despite the different pathogenesis, CF and COPD share common pathological features, such as compromised CFTR function, impaired mucus clearance and airway acidification. The latter could be due to defective bicarbonate transport through the CFTR anion channel. Therefore, we hypothesized that bicarbonate-containing aerosols could be beneficial for patients with CFTR dysfunctions. Our aim was to test the safety and efficacy of chronic bicarbonate inhalation in a COPD animal model, as a model for impaired CFTR function.

3. Methods

3.1. Effect of extracellular pH, ATP and divalent cations on the intracellular Ca^{2+} level of airway epithelial cells

The CFTR expressing human airway epithelial cells (CFBE41o−) were stably transfected with either wild-type or delF508 CFTR cDNA and selected by puromycin resistance. CFBE41o− cells were loaded with Fluo-3/AM (4 μM) in the Na–Ca–7.4-sol for 60 min at room temperature. The cells were then washed with Na–Ca–7.4-sol, and the coverslips were mounted into a perfusion chamber equipped with an inverted microscope. Recordings were made with a confocal laser scanning microscope (Axiovert 200M Zeiss LSM 510 Meta) equipped with a 20× Plan Apochromat (NA = 0.80) DIC objective which is able to acquire data in real-time enabling Ca^{2+} dynamics of individual cells in vitro. We used a 488-nm argon-ion laser for the excitation. The emitted light was collected with BP 505–570 band pass filter. At the beginning of each experiment, we superfused the cells with a standard solution (Na–Ca–7.4-sol) containing NaCl 145 mM, KCl 5 mM, $CaCl_2$ 3 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 10 mM, (pH 7.4). Sodium was substituted with either equimolar N-methyl-d-glucamine (NMDG–Ca–7.4-sol) or lithium (Li–Ca–7.4-sol). The Ca^{2+} depleted solutions were prepared by omitting $CaCl_2$ (Na–7.4-sol or NMDG–7.4-sol). The effects of alkaline external conditions were tested at pH 7.9. In some experiments, to empty intracellular Ca^{2+} stores 0.1 M thapsigargin was used for 15 min prior to the recording. Changes in intracellular calcium concentration ($[Ca^{2+}]_i$) are displayed as the percentage of fluorescence relative to the intensity at the beginning of each experiment. Baseline fluorescence (100%) was calculated from the average fluorescence at the ROIs during perfusion of the cells with Na–Ca–7.4-solution. The autofluorescence was

subtracted from the readings by measuring a cell-free area on the same coverslip. The velocity of changes in $[Ca^{2+}]_i$ was estimated by the slope of the curves (% / min). The average results are expressed as mean ± standard error of the mean (S.E.M.) and each n represents the mean of a single coverslip containing at least 20 cells. Statistical comparisons were made using F-probe and Student's paired t-test. Differences were considered statistically significant when $p<0.05$. For single cell Ca^{2+} measurement cells were plated onto 25-mm diameter circular glass coverslips at a density of 3 × 105 cells / 35-mm wells on day before transfection with plasmid DNA of the mRFP-IP3 R-LBD constructs (1 g/dish) using the Lipofectamine 2000 reagent (Invitrogen) and OPTI-MEM (Invitrogen). On day after transfection cells were loaded with Fura-2/AM (2 μM, 45 min) at room temperature in a modified Krebs–Ringer buffer, pH 7.4 in the presence of 200 M sulfinpyrazone and 0.04 % pluronic acid. Ca^{2+} measurements were performed at room temperature in NMDG solution containing no Ca^{2+}. An inverted microscope (Axio Observer, Zeiss) equipped with a 40× oil immersion objective (Fluar, Zeiss) and a Cascade II camera (Photometrics) was used. Excitation wave-lengths were set by a random access monochromator connected to a xenon arc lamp (DeltaRAM, Photon Technology International). To identify cells expressing mRFP-IP3R-LBD, mRFP fluorescence was measured at the beginning of each experiment using an excitation wavelength of 570 nm with a 610 nm dichroic filter and a 640 nm filter set. For ratiometric measurements of Fura-2 excitation wave-lengths of 340 and 380 nm were selected combined with a 505 nm dichroic filter and a 525/536 nm emission filter set. Data acquisition and processing were performed by the MetaFluor (Molecular Devices) software. Images were acquired every 5 s for a period of 5 min. For time resolved measurement of fluorescence, 340/380 nm ratio value was calculated from the background subtracted recordings. For the immunofluorescent staining the

primary antibodies used were goat polyclonal anti-P2X4, anti-P2X5 and anti-P2X6 (Santa Cruz Biotechnology Inc.). For the double labeling we used rabbit polyclonal anti-P2X4, anti-P2X5 and anti-P2X6 (Alomone Laboratories Ltd.) together with the above-mentioned antibodies. After washing in PBS, we used FITC conjugated rabbit anti-goat secondary antibody (Vector Laboratories Inc.) (dilution: 1:200 in PBS). After washing, we used a mounting media containing DAPI or Propidium iodine for the nuclear staining. (Vector Laboratories Inc.). The samples were then visualized, and pictures were taken with the Zeiss laser scanning microscope.

3.2. Effect of HCO_3^- inhalation in COPD animal model

Experiments were performed on 8-week-old male guinea pigs weighing 600±150 g at the beginning of the study. All procedures were performed in accordance with the 40/2013 (II.14.) Government Regulation on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments and Directive 2010/63/ EU of the European Parliament. They were approved by the Animal Welfare Committee of the University of Pécs and the National Scientific Ethics Committee on Animal Research of Hungary (licence No.: BA02/2000–4/2019 issued on 29 Jan 2019 by the Government Office of Baranya County). Guinea pigs were divided into 4 groups (4 animals / group); 2 groups treated with hypertonic NaCl (8.4 % corresponding to 1.44 M) and the other 2 groups with hypertonic $NaHCO_3$ (8.4 % corresponding to 1 M) aerosol for 30 min, twice daily, 5 days/week, for 8 weeks. The treatment groups were subdivided into groups inhaling only NaCl or $NaHCO_3$, and groups exposed to cigarette smoke besides the respective aerosol treatments. Cigarette smoke exposure (CSE) was performed after aerosol treatment in a whole-body smoke exposure chamber (Teague Enterprise, USA) for 30 min

followed by a ventilation period of 30 min twice daily, 10 times/ week for 8 weeks with the use of 2 research cigarettes at a time (3R4F Kentucky Research Cigarette; University of Kentucky, USA). Body weight was measured daily, respiratory functions (breathing frequency, tidal volume, minute ventilation, inspiratory and expiratory times, peak inspiratory and expiratory flows, as well as baseline enhanced pause correlating with airway resistance) were assessed at the beginning and at the end of week 2, 4, 6 and 8. At the end of the experimental protocol animals were anesthetized by pentobarbital sodium (1 % Euthanimal 400 mg/ml, Alfasan, the Netherlands; 0.5 ml/100 g) and arterial blood was collected for blood gas and acid base analysis by Astrup's equilibration technique in the Department of Laboratory Medicine, University of Pécs, Hungary. Other laboratory parameters were measured from heparinized venous blood by an AU5800 clinical chemistry analyzer (Beckman Coulter Hungary, Budapest, Hungary) in the Department of Laboratory Medicine, Semmelweis University, Budapest, Hungary. Urine pH was assessed every week of the experimental protocol. Three different localizations (apex, hilus, base) were excised from the lungs and fixed in 6 % formaldehyde solution for histopathological assessment. Ten non-cartilaginous airways, ten vessels and ten septa (examined by high power field) were selected from each lung site of every group (equally chosen from each animal). Acute inflammatory cell infiltration (eosinophil and neutrophil granulocytes) was counted in the airways, vessels and septa. Airway intraluminal perimeter was measured (to normalize airway dimensions) with Case Viewer software (3DHISTECH Ltd, Hungary) after scanning each slide with 20× objective (Pannoramic 250 FLASH III scanner, 3DHistech Ltd., Hungary). Statistical analysis was performed by GraphPad Prism v6 software (GraphPad, San Diego, CA, USA). Respiratory parameters and body weight were analyzed by repeated measures two-way ANOVA followed by Tukey's

multiple comparisons test. Histopathological and laboratory parameters were assessed by Kruskal–Wallis followed by Dunn's multiple comparisons test.

4. Results

4.1. Regulation of P2X-mediated calcium entry by extracellular ionic environment

4.1.1. *Purinergic receptor stimulation elicits similar Ca^{2+} signals in wild-type-CFTR and F508del-CFTR expressing cells (CFBEo-)*

In both ΔF-CFTR and wt-CFTR cells, ATP (100 μM) stimulated a biphasic increase in $[Ca^{2+}]_i$. An initial Ca^{2+} peak was followed by a sustained increase of $[Ca^{2+}]_i$. Furthermore, ATP elicited a monophasic response in Ca^{2+}-depleted extracellular saline suggesting that Ca^{2+} entry from the extracellular space was necessary for the sustained phase of Ca^{2+} signal. To test whether extracellular alkalinization would stimulate Ca^{2+} entry, we raised the pH of the bath solution from 7.4 to 7.9 prior to the administration of ATP (100 μM). Under these conditions we observed neither in ΔF-CFTR nor in wt-CFTR cells potentiation of the ATP-induced Ca^{2+} signal. However, ATP (100 μM) elicited a significantly higher sustained Ca^{2+} signal when external pH was raised (7.9) with parallel substitution of extracellular Na^+ by a non-permeant large organic cation NMDG in both ΔF-CFTR and wt-CFTR cells. These data suggested that changes in the external ionic environment modify ATP-induced Ca^{2+} signals independent of the CFTR mutations.

4.1.2. *Effects of Zn^{2+} on ATP-induced Ca^{2+} signal in airway epithelial cells expressing wild-type or F508del-CFTR*

Zn^{2+} is known to interact with several Ca^{2+} entry channels such as P2XRs and SOCs. Depending on P2X receptor subtypes, Zn^{2+} can either potentiate or inhibit ATP-induced Ca^{2+} entry whereas SOCs are mainly inhibited by metal ions. To our surprise in NaCl-rich (145 mM) external solution, $ZnCl_2$ (20 μM) inhibited the ATP-induced sustained Ca^{2+} plateau both in wt-CFTR and ΔF-CFTR expressing cells while peak increase of $[Ca^{2+}]_i$ induced by ATP was not influenced by Zn^{2+}. Increasing the pH from 7.4 to 7.9 suspended inhibitory effects of Zn^{2+} on ATP-elicited sustained Ca^{2+} signal. The prolonged Ca^{2+} plateau is due to the new balance between enhanced Ca^{2+} influx and Ca^{2+} sequestration/extrusion mechanisms. Thus, we hypothesized that the effects of Zn^{2+} could be explained by inhibition of either ATP-gated P2XRs or SOCs activated by ATP-induced intracellular Ca^{2+} store depletion. Store-operated Ca^{2+} channels have been previously shown to be inhibited by Zn^{2+}. Therefore, we presumed that in NaCl-rich environment Zn^{2+} inhibited SOCs rather than P2XRs. Furthermore, Zn^{2+} has been shown to interact with plasma membrane Ca^{2+} ATPase (PMCA) in human erythrocytes. Therefore, we were faced with two possibilities that Zn^{2+} either: (i) inhibited SOC-mediated Ca^{2+} entry or (ii) stimulated PMCA activity. To exclude the latter mechanism, we have pretreated the wt-CFTR expressing cells with thapsigargin that is known to empty intracellular Ca^{2+} stores activating SOCs. In Ca^{2+}-depleted extracellular saline, administration of $BaCl_2$ (3 mM) caused an increase in fluorescence due to Ba^{2+} entry which was arrested by Zn^{2+}. Pretreatment and co-administration of $ZnCl_2$ (20 μM) further slowed down Ba^{2+} entry while removal of Zn^{2+} regained Ba^{2+} influx. Since Ba^{2+} is not a substrate of PMCA, these data suggest that zinc inhibits SOCs rather than influences PMCA activity in CFBE41o− cells. It had been shown that extracellular Na^+ directly inhibited P2XRs and might also compete with Ca^{2+} in the passage through these channels. Therefore, we investigated the effects of Zn^{2+} on ATP-induced

Ca^{2+} signal following changes of extracellular Na^{+} concentration. Furthermore, since ion currents through P2XRs are influenced by external proton concentration, we also tested the effects of ATP and Zn^{2+} at different external pH. In sodium-free, NMDG-containing solution, Zn^{2+} significantly increased the ATP-induced Ca^{2+} plateau at both pH 7.4 and 7.9 but did not at pH 6.9. The complete replacement of sodium is a non-physiological condition; therefore, we tested the effects of zinc at different extracellular Na^{+} levels. Our data showed that zinc increased ATP-induced Ca^{2+} signal but only when the Na^{+} concentration was 40 mM or less.

4.1.3. *Effects of Zn^{2+} and other divalent metal cations on $[Ca^{2+}]_i$ in CFBE41o− cells expressing wt-CFTR*

We have previously shown that, in IB3-1 cells, Zn^{2+} elicited a Ca^{2+} signal in a Na^{+}-free, alkaline environment. Therefore, we tested the effects of $ZnCl_2$ (20 μM) without co-administration of ATP. It is important to note that these experiments were performed only in wt-CFTR cells because Zn^{2+} elicited similar changes in ATP-induced Ca^{2+} signal in both wt-CFTR and ΔF-CFTR expressing cells. In Na^{+}-containing solution Zn^{2+} did not change $[Ca^{2+}]_i$. However, in NMDG-containing solutions, Zn^{2+} elicited a sustained increase in $[Ca^{2+}]_i$. The zinc-induced Ca^{2+} plateau was prevented by removal of Ca^{2+} from the external solution whereas the peak of Ca^{2+} signal was reduced by phospholipase C (PLC) inhibitor, U73122 (10 μM). Since U73122 had statistically significant but fairly small effects, we used another approach to confirm the involvement of PLC in Zn^{2+}-induced Ca^{2+} release from internal stores. The N-terminal IP3 binding region of the type-I IP3R was fused to the mRFP for expression in CFBE41o− cells. In Ca^{2+}-depleted solution, Zn^{2+} evoked Ca^{2+} response was significantly reduced in cells expressing this domain if compared with the non-expressing

control cells. These data indicate the involvement of the PLC and IP3 pathway in this process. To demonstrate whether PLC-dependent Ca^{2+} release could be elicited by other heavy metal divalent cations, we investigated the effects of Ni^{2+} and Cu^{2+}. Similarly to results obtained with Zn^{2+}, administration of $NiCl_2$ (20 μM) caused no change in resting $[Ca^{2+}]_i$ in NaCl-rich medium. Interestingly, $CuCl_2$ (20 μM) elicited a slow and constant increase in the level of $[Ca^{2+}]_i$ suggesting that under these conditions Cu^{2+} induced an irreversible cell damage. However, in the NMDG-containing solution, both Ni^{2+} and Cu^{2+} induced a sustained and reversible Ca^{2+} signal. Following the removal of extracellular Ca^{2+} both cations caused a transient Ca^{2+} signal suggesting that intracellular Ca^{2+} stores were involved. Therefore, we speculate that CFBE41o− cells possess G-protein coupled divalent cation-sensing receptors. However, it is noteworthy that external Na^+ prevents changes of cytosolic Ca^{2+} concentration induced by all the heavy metal cations tested. To investigate whether ATP release during experimental procedure could contribute to Zn^{2+}-induced Ca^{2+} signal, we used the ATP "scavengers" hexokinase and apyrase. Simultaneous application of hexokinase (5 U/ml) and apyrase (1 U/ml) did not prevent effects of Zn^{2+} suggesting that ATP was not involved in Zn^{2+}-induced Ca^{2+} signal. In order to demonstrate that extracellular Na^+ specifically inhibits divalent cation-induced Ca^{2+} signal, we administered Zn^{2+} in a LiCl-rich solution (Na^+-free environment). Under these conditions Zn^{2+} induced an increase in $[Ca^{2+}]_i$.

4.1.4. *Effects of suramin on Zn^{2+}-induced sustained Ca^{2+} signal*

Silberberg and his colleagues have shown that rabbit airway ciliated cells express P2X purinergic receptors which are modulated by extracellular Na^+. In addition, previous observations suggest that P2X4 and P2X6 may co-assemble on

the apical surface of airway epithelial cells. To investigate whether the effects of Zn^{2+} are at least partially mediated by P2XR subtypes, we used a non-specific purinergic receptor antagonist, suramin. Although suramin is a weak or even ineffective inhibitor of homomeric P2X4, it can still effectively block heteromeric P2X4/6 receptors in low micromolar concentrations. In wt-CFTR expressing cells, suramin (10 μM) significantly reduced the sustained increase of Fluo-3 fluorescence elicited by Zn^{2+}.

4.1.5. *Immunoreactivity of P2X4, P2X5 and P2X6 receptor subtypes*

Due to the lack of specific agonists and antagonists, our experiments did not distinguish among the P2X receptor subtypes that are possibly involved in ATP and Zn^{2+}-induced Ca^{2+} entry mechanisms. Therefore, immunohistochemical techniques were used to test whether P2X4, P2X5 and/or P2X6 receptors were present in CFBE41o− cells transfected with ΔF-CFTR. Strong immunohistochemical labeling was detected with the anti-P2X4 and anti-P2X5 as well as anti-P2X6 receptor antibodies. Simultaneous staining of the P2X4/P2X5 and P2X4/P2X6 receptors showed cytoplasmic co-labeling suggesting that these receptor channels might participate in Zn^{2+}-induced Ca^{2+} entry. Similar results were obtained in cells transfected with wt-CFTR.

4.2. Safety and efficacy of chronic hypertonic bicarbonate inhalation in a COPD animal model

4.2.1. Long-term $NaHCO_3$ inhalation improves some CSE-induced transient respiratory alterations

Frequency, inspiratory and expiratory times, as well as peak inspiratory flow showed mild and transient significant alterations in response to CSE throughout the 8-week-long experimental protocol. In the 8.4 % NaCl + CSE-treated group frequency and peak inspiratory flow significantly decreased, while inspiratory time increased compared to the non-smoking respective controls at the end of week 4. These alterations were counteracted by 8.4 % $NaHCO_3$ treatment in CSE animals. The protective effect of $NaHCO_3$ is also supported by the facts that i) in contrast to the NaCl-treated animals, no significant differences developed in any parameters of the $NaHCO_3$ + CSE-treated guinea pigs in comparison with the respective controls, and ii) the inspiratory and expiratory times were significantly shorter in the $NaHCO_3$ + CSE group compared to the NaCl + CSE animals at weeks 2 and 6. There were no changes in tidal volume, baseline enhanced pause and peak expiratory flow in any groups. At the end of the treatments no differences were revealed in the parameters of different experimental groups.

4.2.2. Histopathological changes

Chronic hypertonic $NaHCO_3$ inhalation did not induce significant eosinophil or neutrophil granulocyte infiltration measured in the non-cartilaginous airways, vessels and septa of the lung. There was no decreased airway intraluminal perimeter either in the non-smoking or CS-exposed groups quantified by the Case Viewer software (3DHISTECH Ltd, Hungary). There were some lymphoid follicles sporadically observed in the lung

sections of NaCl -, NaCl + CSE -, as well as $NaHCO_3$- treated guinea pigs.

4.2.3. *Laboratory parameters*

Parameters of electrolyte balance, such as sodium and chloride; creatinine referring to kidney and albumin, total protein, bilirubin, alkaline phosphatase as well as alanine transaminase levels indicating liver functions were within the normal range. Hyperkalemia observed in all groups might be due to hemolysis on blood collection. Chronic inhalation of hypertonic $NaHCO_3$ did not induce metabolic alkalosis, the alkaline urine pH characteristic of herbivores was within the physiologic range. The arterial blood gas analysis performed before tissue harvesting revealed acute respiratory acidosis with elevated P_aCO_2 and low arterial pH that could be most likely due to pentobarbital anesthesia-induced respiratory depression. The body weight gain of the animals was affected neither by hypertonic $NaHCO_3$ aerosol treatment nor by CSE.

5. Conclusions

Investigating the calcium homeostasis of human airway epithelial cells in vitro and studying the effects of inhalation of hypertonic $NaHCO_3$ in vivo, I obtained the following results: (i) the parallel removal of Na^+ and alkalinization of the extracellular environment significantly enhanced both the amplitude and duration of ATP-induced Ca^{2+} signal in human airway epithelial cells independent of CFTR protein expression, (ii) Zn^{2+} either potentiates or inhibits ATP-induced Ca^{2+} entry from the extracellular space depending on the absence or presence of extracellular Na^+, (iii) Zn^{2+} and other heavy metal cations stimulate release of Ca^{2+} from internal stores via the PLC-dependent pathway only in Na^+-free environment, (iv) there are multiple subtypes of P2X receptor channels on human airway epithelial cells which might be involved in the divalent metal cation-induced Ca^{2+} entry mechanisms, (v) 8-week-long inhalation of hypertonic $NaHCO_3$ is as safe as NaCl in cigarette smoke-induced airway irritation guinea pig model. These findings indicate that purinergic agonists applied to the airways in an aerosol containing HCO_3^- and Zn^{2+} with low Na^+ concentration (40 mM or less) should be considered potentially valuable therapeutic approach is chronic inflammatory airway diseases such as CF and COPD. Taken together, data presented here underline the importance of aerosol composition that could further potentiate the efficacy of purinergic agonists.

6. Bibliography of the candidate's publications

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