Role of extracellular zinc in the regulation of ion transport in airway and intestinal epithelium

Ph.D thesis

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1. Introduction

Zinc is one of the most widely utilized biometal in the human body because of its physical and chemical properties that enables it to interact with a variety of proteins and enzymes. Zn^{2+} is an important component of the catalytic site of several metalloenzymes, and also a crucial structural component of hundreds of "zinc finger"-containing proteins. Furthermore, Zn^{2+} is an allosteric modulator of a wide variety of ion channels, potentiating or inhibiting their activity.

Epithelial cells form dynamic interfaces between different physiological compartments. They share common characteristics as covering and protecting surfaces exposed to injury by providing a physical barrier. Furthermore, they maintain tissue homeostasis by allowing the regulated transport of solutes, electrolytes and water across them.

The transport of Na^+ and Cl^- by airway epithelium is essential for the maintenance of mucociliary clearance that represents the primary protective mechanism against infections in the airways. Cystic fibrosis (CF) airway epithelium exhibits reduced capacity to secrete Cl^- and/or HCO_3^- and also increased Na^+ absorption, due to the absence or dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) channel. This leads to the dehydration of the airway surface liquid (ASL), which is incompatible with efficient mucus transport. Since lung manifestations are the dominant source of morbidity in CF, several approaches have been considered with the potential to delay or arrest the development of CF lung disease. The Ca^{2+}-activated Cl^- channel (CaCC) has often been referred to as the alternative Cl^- channel, because it may provide a parallel route for Cl^- secretion across the apical membrane in tissues that lack CFTR. Therefore, pharmacotherapeutic interventions aimed to stimulate alternative CaCCs are

under development. Previously we have demonstrated that extracellular Zn^{2+} regulates purinergic Ca^{2+} influx in human CF and non-CF airway epithelial cells which ability is strongly dependent on the pH and Na^{+} concentration of the extracellular environment. Furthermore, low micromolar Zn^{2+} (20µM) when applied to an alkaline, low Na^{+}-containing solution ("enhanced calcium transfer" solution) produced a Ca^{2+} signal and Cl^{-} secretion in CF airway epithelial cells comparable to that when Zn^{2+} was co-administered with ATP. Thus, the administration of Zn^{2+} in a properly composed aerosol might be advantageous for CF patients by improving MCC function.

The Ca^{2+} homeostasis of the human body is achieved by the concerted action of three organ systems including the gastrointestinal tract, bone, and kidney. Ca^{2+} is absorbed from the lumen of the intestines via the apical epithelial calcium channel transient receptor potential vanilloid type 6 (TRPV6), facilitated by both chemical and electrical gradients. The TRPV6 channel is ligand independent, it is not sensitive to capsaicin, heat or osmotic stress. Its pivotal role is to form constitutively active Ca^{2+} entry pathways in Ca^{2+} transporting epithelia including intestinal and placental epithelium. The most important regulators of TRPV6 expression are the biologically active form of Vitamin D_3 [$1,25(OH)_2D_3$], estrogen and dietary Ca^{2+} intake. Normal adults require approximately 10-15 mg/day Zn^{2+} intake, which mostly vary as a function of age and excretion. Zn^{2+} is absorbed in the small intestines, mainly in the jejunum and ileum. Absorption is accomplished by both passive diffusion and through different zinc-specific transporters such as the ZIP4 (Zrt- and Irt-like protein 4, SLC39 family). Recently, the TRPV6 transporter protein has been proposed to be involved in Zn^{2+} absorption. There are a few studies reporting that the ancestral form of mammalian TRPV6 in fish also transports Zn^{2+}. Rat

TRPV6 expressed in *Xenopus* oocytes was reported to transport Ca^{2+}, Ba^{2+}, Sr^{2+} but not Mg^{2+} or any of the other di- or trivalent cations examined. When human TRPV6 (hTRPV6) was studied in the same system, similar results were obtained. To date, no studies are available investigating the permeability of human TRPV6 in mammalian expression systems, or of endogenous TRPV6 to different trace elements and toxic heavy metals.

2. Specific aims

The main purpose of the present work was to elucidate the effects of extracellular zinc on Ca^{2+} entry mechanisms in two different cellular models. The specific aims were as follows:

1. To investigate the role of zinc in different extracellular ionic milieu on the activity of Ca^{2+}-dependent Cl^- channels in CF airway epithelial cells.

2. To study the effect of extracellular zinc on human TRPV6, an intestinal Ca^{2+} transporter channel expressed in HEK293 cells.

3. Materials and methods

3.1. Cell culture protocols

The CF human bronchial epithelial cell line IB3-1 was grown in DMEM/F12 (1:1) medium. The human embryonic kidney 293 cell line (HEK293) was cultured in DMEM cell culture media. Both cell lines were maintained at 37^{o}C in a humidified cell culture incubator supplied with 5% CO_2.

3.2. Transfection protocol for HEK293 cells

Cells were plated at 300,000 cells/well density on No. 00 coverslips coated with 100µg/ml poly-D-lysine in 35 mm dishes. After 8-16 hours, cells were transfected with 2 µg pEYFP-C1-TRPV6 using 5 µl Lipofectamine 2000 per dish. For control experiments, cells were transfected with the pEYFP-C1 vector. In some experiments, cells were transfected with 2 µg pTagRFP-C1-hTRPV6 construct to avoid the interference of EYFP and fluorescence dyes with similar spectra.

3.3. Cytosolic Ca^{2+} measurement in IB3-1 airway epithelial cells

Cells were loaded with Fluo-3-acetoxymethyl ester (AM, 4 µM) in standard extracellular solution for 60 minutes at room temperature. Standard extracellular solution contained (in mM): 145 NaCl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 mM HEPES, pH 7.4 (with NaOH). Sodium-free solutions contained (in mM): 145 NMDG-Cl, 5 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 mM HEPES, varying pHs were adjusted by either NMDG or HCl. Nominally Ca^{2+}-free solutions were prepared by simply omitting $CaCl_2$. Recordings were made with a confocal laser scanning microscope, Axiovert 200M Zeiss LSM 510 Meta. Changes in $[Ca^{2+}]_i$ are

displayed as the percentage of fluorescence relative to the intensity at the beginning of each experiment.

3.4. Cytosolic divalent metal cation measurement in hTRPV6 expressing HEK293 cells

For the detection of Ca^{2+} or other divalent cation entry, non-transfected and transfected cells on the same piece of coverslip were loaded with 5 µg/ml Fura-2-AM (stock solution 5 µg/µl) for one hour in serum-free medium in a cell culture incubator at 37 °C. To confirm that the change in fluorescence is due to an increase in intracellular Zn^{2+} levels rather than Ca^{2+} or Mg^{2+} levels, we loaded the cells with 5 µM NewPort Green DCF (stock solution 5 mM) for 45 minutes. Thereafter, cells were placed into modified Krebs-Ringer HEPES (KRH) (150 mM NaCl, 4.8 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES, pH 7.4). Fura-2 was alternatingly excited at 340 nm and 380 nm, and the F340/F380 ratio was monitored at the emission wavelength of 510 nm. The initial rate of the increase of the Fura-2 ratio was taken as the measure of the influx of the particular cation. NewPort Green DCF was excited at 500 nm, and the initial increase of the fluorescence intensity in response to the addition of the specific cation was measured. Fluorescence measurements were performed on a Nikon Eclipse TiU inverted microscope equipped with a polychrome V+ light source.

3.5. Electrophysiology

Voltage-clamp recordings were carried out in the standard whole-cell configuration using an Axopatch 200B amplifier and a Digidata 1200 interface board. Micropipettes were pulled by a P-97 Flaming-Brown type micropipette puller from borosilicate glass capillary tubes and had a tip resistance of 3–6 MΩ when filled with pipette solution. Command protocols

and data acquisition were controlled by pClamp 6.03 software. Solutions were delivered by continuous perfusion with a gravity-fed delivery system.

3.5.1. Whole cell experiments in IB3-1 airway epithelial cells

Currents were monitored at -80 mV using ramp commands (-100 mV to +100 mV in 200 ms, 1mV/ms) applied every 10 s. The holding potential was -50 mV between ramps. All reported currents were normalized by cell capacitance and expressed as current density (pA/pF). Standard pipette solution contained (in mM): 140 NMDG-Cl, 1 $MgCl_2$, 2 EGTA, 10 HEPES, pH 7.2 (with NMDG) and an appropriate concentration of $CaCl_2$, to give free $[Ca^{2+}]_i$ = 0.1 µM. In some experiments high free $[Ca^{2+}]_i$ = 1 µM was used. Free $[Ca^{2+}]_i$ was estimated using MaxChelator software. Low intracellular Cl^- solution contained (in mM): 90 NMDG-glutamate, 50 NMDG-Cl, 1 $MgCl_2$, 2 EGTA, 10 HEPES, pH 7.2 with NMDG (free $[Ca^{2+}]_i$ = 0.1 µM). Increased Ca^{2+}-buffering pipette solution contained (in mM): 140 NMDG-Cl, 1 $MgCl_2$, 20 EGTA, 10 HEPES, pH 7.2 (with NMDG). Standard extracellular solution contained (in mM): 145 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 10 HEPES, pH 7.4 (with NaOH). Na^+-free control extracellular solution contained (in mM): 145 NMDG-Cl, 1 $MgCl_2$, 10 D-glucose, 10 HEPES, pH 7.4 (with NMDG) and 3 $CaCl_2$, unless stated otherwise. Niflumic acid (NFA) was added to the bath solution for 5 min before initiating any experimental protocol.

3.5.2. Whole cell experiments in hTRPV6 expressing HEK293 cells

Voltage clamp recordings were carried out using ramp commands (-110 mV to +90 mV in 200 ms, 1mV/ms) applied every 5 seconds. The holding potential was -20 mV between ramps. Patch pipette filling solution contained (in mM): 140 NMDG, 1 $MgCl_2$, 20 EGTA and 10 HEPES,

adjusted to pH 7.2 with HCl. Normal extracellular solution contained (in mM): 147 NaCl, 3 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, 10 HEPES, adjusted to pH 7.4 with NaOH. Control solution with no divalent cations contained (in mM): 147 NMDG, 15 D-glucose, 10 HEPES, adjusted to pH 7.4 with HCl. During recordings, the bath solution was changed to a control solution supplemented with 2 mM test divalent cation (XCl_2; X = Ca^{2+}, Zn^{2+}, Cd^{2+}).

3.6. ^{45}Ca uptake assay in HEK293 cells

Radioactive uptake assays were performed 48 hours after transfection, and the uptake rates were always measured in parallel for transfected and control cells. The cells were exposed for a set time to 2 µCi ^{45}Ca in KRH solution containing 100 µM cold calcium with or without zinc. Calcium uptake was stopped by rinsing the cells with ice-cold KRH-solution.

3.7. Data presentation

Results were presented as means ± SEM of N observations. Statistical significance was determined using paired Student's t-test or the non-parametric rank sum t-test. Differences were considered statistically significant when $p < 0.05$.

4. Results

4.1. Extracellular pH and zinc regulate cytosolic [Ca^{2+}] and CaCC activity in CF airway epithelial cells

In previous studies it was shown that modifications of the extracellular ionic environment (Na^+ and/or H^+) might influence cytosolic Ca^{2+} levels. Therefore first we investigated the effects of extracellular alkalinization on $[Ca^{2+}]_i$. In the presence of Na^+ (145 mM) alkalinization of pH_o (from 7.4 to 7.9 or 8.2) did not cause significant alterations in basal $[Ca^{2+}]_i$ (NaCl pH_o 7.4: 101.7 ± 0.7 % [N=16] vs. NaCl pH_o 8.2: 101.8 ± 2.3 % [N=5] p=n.s). In subsequent experiments, extracellular Na^+ was substituted by a non-permeant large organic cation N-methyl-D-glucamine ($NMDG^+$). At pH_o 7.4, acute removal of external Na^+ did not cause a change in basal Ca^{2+} level (NaCl pH_o 7.4: 101.7 ± 0.7 % [N=16] vs. NMDG-Cl pH_o 7.4: 101.9 ± 1.1 % [N=5] p=n.s). However, when the pH_o was raised to 7.9 with parallel replacement of extracellular Na^+, a mild but sustained elevation in intracellular Ca^{2+} levels could be observed (NaCl pH_o 7.4: 101.7 ± 0.7 % [N=16] vs. NMDG-Cl pH_o 7.9: 133.5 ± 3.5 % [N=5] $p < 0.05$). Raising pH_o to an even higher level (from 7.4 to 8.2) elicited a markedly greater increase in intracellular calcium levels that reached a plateau within the first 3 minutes (NaCl pH_o 7.4: 101.7 ± 0.7 % [N=16] vs. NMDG-Cl pH_o 8.2: 239.8 ± 13.3 % [N=5] $p < 0.05$). To investigate whether the increase in $[Ca^{2+}]_i$ was due to Ca^{2+} entry, we repeated the experiments with nominally Ca^{2+}-free solutions. Under these experimental conditions cells failed to respond with an increase in $[Ca^{2+}]_i$ (3 mM $CaCl_2$: 239.8 ± 13.3 % [N=5] vs. Ca^{2+}-depleted solution: 107.6 ± 1.1 % [N=5] $p < 0.05$ at pH_o 8.2 in the presence of NMDG-Cl) suggesting that Ca^{2+} originated from the extracellular space.

In contrast to the effects of extracellular alkalinization, lowering the pH_o (from 7.4 to 6.6) in the absence of extracellular Na^+ caused a mild, but sustained decrease in $[Ca^{2+}]_i$ (NaCl pH_o 7.4: 101.7 ± 0.7 % [N=16] vs. NMDG-Cl pH_o 6.6: 85.9 ± 2.4 % [N=5] $p < 0.05$).

To investigate whether the alkaline pH-induced Ca^{2+} signal could stimulate chloride efflux in these airway epithelial cells, we used the patch clamp technique in whole cell configuration. Control currents were recorded following substitution of external Na^+ by $NMDG^+$. At pH_o 8.2, we observed a slowly activating, large inward current that reached a plateau in approx. 2 min. This current was fully reversible upon resetting the pH_o to 7.4, and reversed near the equilibrium potential of Cl^- ($E_{rev.}$= -3.2 ± 0.7 mV [N=8] vs. E_{Cl^-} = -1.9 mV). Following partial replacement of intracellular Cl^- with glutamate, the reversal potential of the alkaline pH_o-induced current remained close to that of E_{Cl^-} (E_{rev}: -25.9 ± 1.7 mV [N=5] vs. E_{Cl^-}: -28.7 mV). These data suggest that inward currents measured at -80 mV represent chloride efflux. In contrast, acidification of pH_o (6.6) resulted in a slight decrease in inward currents. Since the complete absence of Na^+ is a non-physiological condition, therefore we also tested the effects of alkaline pH_o at various extracellular Na^+ concentrations. External alkalinization did not elicit an increase in inward currents when external Na^+ concentration was near to physiological values. To test whether CaCCs were involved in the alkaline pH_o-induced increase of chloride currents, we first examined the dependency on external calcium. In NMDG-rich solution, at pH_o 8.2 the peak inward current (-11.0 ± 1.4 pA/pF [N=8] at -80 mV) was reduced when experiments were performed either in extracellular solution containing 0.1 mM Ca^{2+} (-2.1 ± 0.1 pA/pF [N=15] $p < 0.05$ at -80 mV) or by the application of 20 mM EGTA in the pipette solution (-1.1 ± 0.1 pA/pF [N=6] $p < 0.05$ at -80 mV). These results indicate that Ca^{2+} entry from

extracellular space plays a crucial role in the activation of the whole-cell inward currents in these CF airway epithelial cells. Additionally, the alkaline pH_o-induced chloride current was prevented by 100 µM NFA, a potent inhibitor of CaCCs.

Our group has extensively investigated the effects of low micromolar Zn^{2+} (20 µM) in IB3-1 cells in the range of pH_o 7.3-7.9. Here we tested the Zn^{2+}-induced Ca^{2+} entry, represented by the plateau phase of the Ca^{2+} signal, at both pH_o 6.6 and 8.2 in the absence of Na^+. At alkaline pH_o (8.2) Zn^{2+} elicited a sustained Ca^{2+} plateau, while at acidic pH_o (6.6) we observed only a transient increase in Ca^{2+} signal. These data indicate that Zn^{2+} promotes Ca^{2+} entry in a pH_o-dependent manner. If cytosolic $[Ca^{2+}]$ were the only factor that determines CaCC activity, application of zinc in alkaline medium would result in large Cl^- currents. To our surprise, however, when cells were perfused with a Na^+ -free, alkaline solution in the presence of $ZnCl_2$ (20 µM), inward currents were transient and the maximal amplitude reached only approx. half the value of the current that we observed in the absence of Zn^{2+}.We hypothesized that this effect of Zn^{2+} was due to direct inhibition of CaCCs. Indeed, application of Zn^{2+} (20 µM) exerted a strong inhibitory effect on steady-state currents induced by high (1 µM) free $[Ca^{2+}]_i$ containing pipette solution. Additionally, the inward current evoked by 1 µM free $[Ca^{2+}]_i$ was prevented by 5 min pretreatment with 20 µM Zn^{2+}. Taken together, these data show that although Zn^{2+} stimulates Ca^{2+} entry in airway epithelial cells it also effectively inhibits CaCCs.

4.2. Zinc regulates and permeates human TRPV6 channels

First, we established a protein expression model to assess the permeability features of hTRPV6 channels. When $[Ca^{2+}]_i$ was estimated with Fura-2,

basal intracellular Ca^{2+} was elevated in EYFP-hTRPV6-transfected HEK cells compared to non-transfected cells (340/380 ratios: 3.227 ± 0.036 (cell number = 682) vs. 1.435 ± 0.01 (cell number = 421). When only EYFP was transfected into the cells, no difference was observed. Removal of extracellular Ca^{2+} almost completely abolished this significant difference (340/380 ratios: 1.1317 ± 0.005 vs. 1.269 ± 0.004). Re-administration of 1 mM Ca^{2+} evoked a much larger increase of $[Ca^{2+}]_i$ in EYFP-hTRPV6-transfected compared to non-transfected cells. In EYFP-transfected cells, the 1 mM Ca^{2+}-induced change was the same in both transfected and non-transfected cells.

Next we aimed to test the permeability of TRPV6 channels for other divalent cations belonging to the calcium group elements (Ba^{2+}, Sr^{2+}). Our data show that when we substituted Ca^{2+} either with Ba^{2+} or Sr^{2+}, both divalent cations entered the cells through TRPV6. This observation is in agreement with results from previous studies.

Since zinc is an essential biometal of particular biological importance, we also attempted to determine whether Zn^{2+} is conducted by human TRPV6 channels. Administration of 1 mM Zn^{2+} caused a significant increase in fluorescence in TRPV6 expressing cells. For comparison, we also tested the permeation properties of TRPV6 for other members of the zinc group elements (Cd^{2+}, Hg^{2+}). These results demonstrated a greatly increased permeability to Cd^{2+} but not to Hg^{2+} in TRPV6 transfected cells.

Furthermore, we examined the effect of Ca^{2+} on Zn^{2+} influx through hTRPV6 using NewPort Green DCF, a calcium-insensitive fluorescence dye. For these measurements, we transfected HEK293 cells with a pTagRFP-C1-hTRPV6 construct to avoid the interference of EYFP and NewPort Green DCF fluorescence due to their very similar spectra. Our

results showed that 1 mM Ca^{2+} significantly blocked the entry of Zn^{2+} (1 mM) through hTRPV6.

To further confirm the previously obtained results with calcium and zinc, we used the patch clamp technique in whole-cell configuration. Divalent cation influx was detected by monitoring inward currents at -80 mV during voltage ramps. Initially, both EYFP-hTRPV6-transfected and non-transfected HEK293 cells were perfused with an extracellular solution lacking charge carriers. Supplementation of the solution with 2 mM Ca^{2+} evoked large, inwardly rectifying currents in EYFP-hTRPV6-transfected cells, while no such current was detected in non-transfected cells.

Next, we found that Zn^{2+} (2 mM) produced a transient current with peak amplitude of 20-100 pA, suggesting that Zn^{2+} also permeates TRPV6. The decay of this transient current may be due to inhibition of TRPV6 by Zn^{2+} itself. Cd^{2+} (2 mM) produced sustained inward currents similar to those observed in the presence of Ca^{2+}.

We found that Zn^{2+} not only permeated but also modulated hTRPV6 channel activity in a biphasic manner. When we pretreated the cells with different concentrations of Zn^{2+}, high concentrations (200 and 2000 µM) inhibited, whereas low concentrations (2 and 20 µM) augmented the inward currents evoked by the administration of 2 mM Ca^{2+}. To confirm our results of the electrophysiological measurements, we also used the ^{45}Ca uptake assay in the next experiments. Our data show that low concentrations of Zn^{2+} induced an increase, whereas high concentrations of Zn^{2+} inhibited ^{45}Ca uptake. These findings are in agreement with the results obtained with patch-clamp technique.

5. Conclusions

1. Zn^{2+} exerts a dual effect on Ca^{2+}-dependent chloride channels (CaCCs) in CF airway epithelial cells. Zn^{2+} indirectly enhanced, however directly blocked the activity of CaCCs. Nonetheless, extracellular alkalinization per se could elicit Ca^{2+} entry and evoke Ca^{2+}-activated Cl^- conductance without the application of Zn^{2+}. These findings suggest that a sufficiently alkaline, Zn^{2+}-free saline aerosol might be also beneficial for CF patients;

2. Zn^{2+} modulates hTRPV6 function in a dose-dependent manner. High concentrations of zinc inhibit, whereas low concentrations enhance Ca^{2+} transport via hTRPV6 channels. These findings suggest that dietary Zn^{2+} intake might play an important role in hTRPV6 channel-mediated transepithelial Ca^{2+} transport in the intestines. Furthermore, the hTRPV6 channels are also permeable for Zn^{2+} in the absence of Ca^{2+}, representing a novel pathway for Zn^{2+} absorption under calcium-restricted conditions.

6. List of publications

6.1. Publications related to the thesis

Dankó T, Hargitai D, Pataki A, Hakim H, Molnar M, and Zsembery A: Extracellular alkalinization stimulates calcium-activated chloride conductance in cystic fibrosis human airway epithelial cells. *Cell Physiol Biochem*, 2011. 27(3-4): p. 401-10.
IF: 3.585

Kovacs G, **Dankó T**, Bergeron MJ, Balazs B, Suzuki Y, Zsembery A, and Hediger MA: Heavy metal cations permeate the TRPV6 epithelial cation channel. *Cell Calcium*, 2011. 49(1): p. 43-55.
IF: 3.553

6.2. Publications not related to the thesis

Hargitai D, Pataki A, Raffai G, Füzi M, **Dankó T**, Csernoch L, Várnai P, Szigeti GP, and Zsembery A: Calcium entry is regulated by Zn2+ in relation to extracellular ionic environment in human airway epithelial cells. *Respir Physiol Neurobiol*, 2010. 170(1): p. 67-75.
IF: 2.382