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DIPLOMARBEIT

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„TNF-α induced activation of different ENaC-subunit combinations in a heterologous expression system“

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVATIONS

DMEM	Dulbecco´s modified Eagle medium
EC_{50}	half maximal effective concentration
eGFP	enhanced green fluorescent protein
E_h	holding potential
hENaC	human amiloride-sensitive epithelial sodium channel
FBS	fetal bovine serum
HEK-293	human embryonic kidney 293 cells
LB	Luria-Bertani
PBS	phosphate buffered saline
pen-strep	penicillin-streptomycin
PHA I	pseudohypoaldosteronism I
rpm	rotations per minute
SOC	super optimal broth with catabolite repression
TIP	lectin-like domain of TNF-α
TNF-α	tumour necrosis factor alpha
wt	wild type

1. Introduction

1.1. The epithelial sodium channel

The epithelial sodium channel (ENaC) is part of the ENaC/degenerin gene family. This family encodes especially sodium channels which are important for several cell functions. The three main subfamilies contain the Caenorhabditis elegans degenerins DEG, the acid-sensing ion channels ASIC as well as the non-neuronal sodium channels SCNN1 genes which are responsible for encoding the four subunits of ENaC (α, β, γ and δ) (Kellenberger et al. 2002).

In contrast to the other family members ENaC is both highly sensitive to sodium and amiloride (Kashlan et al. 2011).

1.1.1. Function and location

ENaC plays an important role for the sodium homeostasis. So it is expressed in many different tissues like in the cell membranes of the colon, lung, kidney and sweat glands (Kashlan et al. 2011). The δ-subunit of ENaC is found in pancreas, testis, ovary and in a low percentage in brain and heart as well (Waldmann et al. 1995). Therefore this channel is jointly responsible for the extracellular fluid volume, the blood pressure and the airway surface liquid volume (Kashlan et al. 2011).

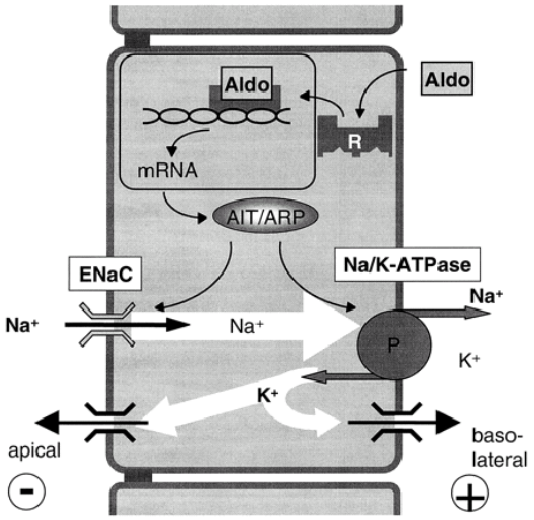

Figure 1: Sodium transport through ENaC in a polarized epithelial cell by aldosterone

Aldosterone (aldo) is bonded to its intracellular receptor and is transported into the nucleus. There it induces the expression of aldosteron-induced (AIT) as well as aldosteron-repressed transcripts (ART). Thereby an increased generation of ENaC and Na^+/K^+-ATPase is caused.

So there is an improved sodium intake from the lumen at the apical side of the cell via ENaC. The Na^+/K^+-ATPase at the basolateral side replaces sodium for potassium to keep the intracellular gradient. Following the electrochemical gradient potassium is secreted through potassium channels which are located at the apical and basolateral side of the cell (Kellenberger et al. 2002).

General ENaC is placed in the apical membrane of polarized epithelial cells and hence it is responsible for the active transcellular sodium transport across tight epithelia (Kellenberger et al. 2002).

In the kidney and the colon this sodium transport is decisive and it is regulated by the mineralcorticoid hormone aldosteron (Figure 1). In contrast the sodium transport in the lung respectively salivery glands is not relevant for the sodium homeostasis of the total body but this transport is necessary for the maintenance as well as the ionic consistence of the airway surface liquid (Kellenberger et al. 2002).

1.1.2. Structure

Four subunits of ENaC (α, β, γ and δ) are known in human tissue. Each subunit is composed of two transmembrane helices M1 and M2 as well as an extensive extracellular loop. The short amino and carboxy termini are located intracellularly (Figure 2) (Kellenberger et al. 2002, Bhalla et al. 2008). The channel pore is made of the transmembrane domain M2 of the α-subunit and of a few extracellular elements from each subunit (Snyder et al. 1999).

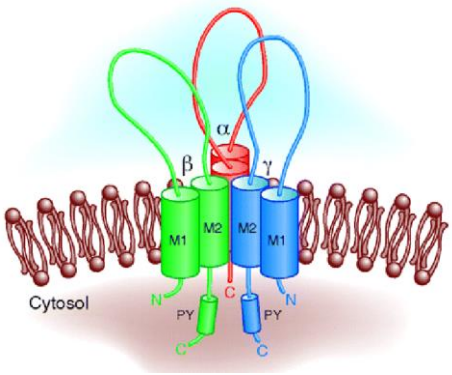

Figure 2: Structure of ENaC (Bhalla et al. 2008)

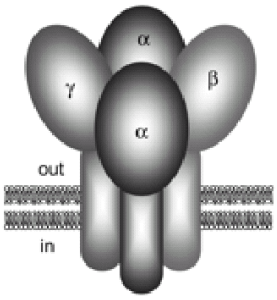

Figure 3: Formation of the subunits of ENaC (Kellenberger et al. 2002)

The combined expression of the α-, β- and γ-subunit causes functional channels (Canessa et al. 1994). The δ-ENaC subunit has a very similar amino acid sequence as the α-subunit. For this reason the δ-subunit is able to replace α-ENaC and to generate efficient channels (Waldmann et al. 1995).

The exact numerical formation of the subunits is still discussed. Based on several biochemical attempts it is supposed that ENaC has a heterotetrameric structure existing of two α-, one β- and and also one γ-subunit (Figure 3) (Firsov et al. 1998, Kosari et al. 1998). But there is also the view that ENaC consists of nine subunits, then three α-, three β- and three γ-subunits (Snyder et al. 1998).

1.1.3. Regulation of ENaC

ENac is managed by diverse intracellular and extracellular factors. So there are different pathways which affect the activity of ENaC via intrinsic ENaC trafficking, regulation of ENaC expression respectivily synthesis and single channel properties as open probability (Garty et al. 1997).
In the cell membrane the amount of the channels depends on their integration and concurrent on their depletion (Eaton et al. 2010). An important role has the ubiquitin ligase Nedd4-2. This enzyme leads to the internalisation of ENaC by binding with prolin rich motifs in the carboxy termini of the β- and γ-subunit (Ruffieux-Daidié et al. 2008, Eaton et al. 2010).
Then the open probability of ENaC which depends on mechanical stress and some other ligands influences also the intensity of the sodium transport (Kashlan et al. 2011).

Furthermore proteolytic cleavage by proteases such as furin can enhance the open probability and is so able to activate ENaC. The α-subunit is cleaved twice and the γ-subunit once by furin in the Golgi vesicles. This cleavage occurs at the extracellular loop in the denoted subunits (Hughey et al. 2004, Sheng et al. 2006). This mechanism does not influence the expression of ENaC at the surface of cell but raises the open probability.

At high intracellular sodium concentrations the sodium current of ENaC is reduced due to a so called negative feedback mechanism (Snyder 2002). In contrast the self-inhibition of ENaC is induced by a high extracellular sodium concentration (Kellenberger et al. 2002).

1.1.4. Diseases based on ENaC

ENaC plays an important role in the human physiology. So many different diseases are associated with mutations or dysfunction of ENaC such as pseudohypoaldosteronism type I (PHA I) or Liddle`s syndrome (Rossier et al. 2002, Bhalla et al. 2008).

PHA I is generated by a reduced or a total loss-of-function mutation of ENaC. The consequence of this mutation is a renal loss of salt and hence symptoms are developed like hypotension, volume deficit, hyperkalemia, hyponatremia and metabolic acidosis as well as increased levels of plasma aldosterone and renin (Kellenberger et al. 2002, Bhalla et al. 2008).
Otherwise there is also a gain-of-function mutation called Liddle`s syndrome. Thus there is a strong sodium intake because of enhanced assembly and activity of ENaC in the cell membrane. This leads to symptoms such as hypertension, volume enhancement, hypokalemia, metabolic alkalosis and a minor level of aldosterone.
Other related diseases to ENaC apply inter alia the lung and the gastrointestinal tract (Knight et al. 2005, Bhalla et al. 2008).

1.2. The tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF-α) is an inflammatory cytokine which is produced by many different kind of cells like macrophages, monocytes and lymphocytes (Fukuda et al. 2001). Especially it is released during acute inflammations (Idriss et al. 2000).
Then TNF-α is involved in a plenty of signalling mechanisms which leads cells either to apoptosis or to necrosis amongst others. Furthermore it is also responsible for the resistance against infections as well as cancer (Idriss et al. 2000).
TNF-α plays also an important role in the up-regulation of the alveolar fluid clearance in alveolar epithelial cells (Baldwin et al. 1996). This mediated increase was studied and it was shown that TNF-α increases the amiloride-sensitive current of ENaC and that the lectin-like domain of TNF-α probably induces this effect (Fukuda et al. 2001). So peptides which are modified from TNF-α can be used in the therapy of pulmonary lung oedema.

Pulmonary lung oedema can be induced by an injury of the alveolar epithelium respectively endothelium or by infections of the lung caused by bacteria or viruses (Matthey et al. 2000, Lucas et al. 2009, Xiong et al. 2010). Furthermore it occurs in the acute respiratory distress syndrome and also in acute lung injury (Dudek et al. 2001).
TNF-α has two opposing effects on the alveolar liquid clearance (Figure 4). On the one side it is responsible for the inflammatory process. On the other side the lectin-like domain of the cytokine is able to accelarate the resorption of the oedema (Braun et al. 2005). This occurs over an increased amiloride-sensitive sodium intake via ENaC which was shown in A549 cells (Fukuda et al. 2001).

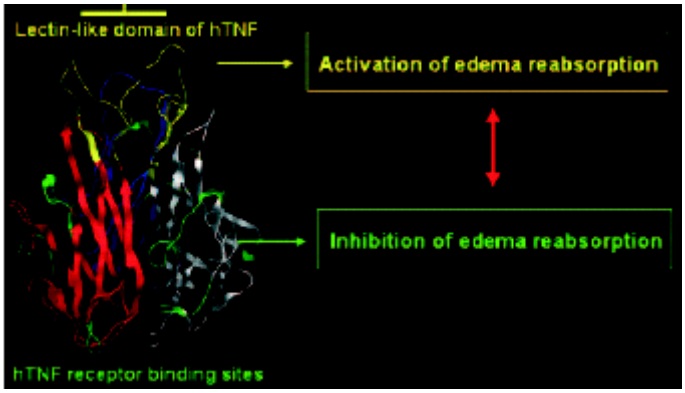

Figure 4: Dual role of TNF-α
in green: receptor binding sites inhibiting oedema resorption
in yellow: lectin-like domain activating alveolar liquid clearance and so also activating oedema resorption
(Braun et al. 2005)

1.3. The lectin-like domain of TNF-α

The lectin-like domain of TNF-α (TIP) stimulates the amiloride-sensitive sodium uptake via ENaC (Fukuda et al. 2001). This structure was the origin to design new peptides which mimic the TIP and activates ENaC (Hazemi et al. 2010).

All designed peptides are smaller than TNF-α and they do not bind to the TNF-α receptor binding sites. Thereby these peptides have no inflammatory effects (Hazemi et al. 2010).

So AP301 was found and it passed the clinical trial phase II. Before it was shown that the inhalation of nebulised AP301 enhanced the alveolar liquid clearance (Hartmann et al. 2013).

Besides AP301 is very selective for ENaC and also enhances the open probability of the channel (Shabbir et al. 2013).

2. Aim of diploma thesis

It was shown that AP301 enhances the open probability of ENaC as well as activates the amiloride-sensitive sodium current depending on the appropriate subunits (Hazemi et al. 2010, Shabbir et al. 2013).

But are these findings only relevant for the TNF-α lectin-like domain derived peptide AP301 or does TNF-α cause similar effects?

3. Material and methods

The response of the different single ENaC subunits on TNF-α was surveyed with the patch-clamp technique in a heterologous expression system.
So HEK-cells were transfected with the relevant cDNA. Following their whole-cell current was determined before and after adding TNF-α to the external solution.

3.1. Cell culture

Cells of the human embryonic kidney, HEK-293, were purchased from American Type Culture Collection (Manassas, VA, USA).
These cells served as a model for a heterologous cell line. They do not express ENaC and so the desired single subunit (α-, β-, γ- or δ-wt) of this channel can be studied.
These cells were used in passages 7 to 26.

All work in the cell culture was performed under aseptic conditions. This included thawing, splitting, seeding and freezing cells.

The cells were cultivated in tissue culture flasks with DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (pen-strep) and given in an incubator with optimal growing conditions (37°C, 5% CO_2).
Cells were surveyed daily under an invert microscope for their morpholgy and their confluence. The cells were split 1:10 at a confluence around 90% for two or three times a week, as described later.

0.05% Trypsin/EDTA with phenol red was used for splitting the cells. Trypsin is responsible for cleaving the proteins which link the adherent cells to the coated bottom of the flask. PBS without Ca^{2+}/Mg^{2+} was used for washing and neutralizing the cells.

3.1.1. Splitting and seeding cells

First the total amount of the media was aspirated from the T25-flask. 1.5 ml PBS without Ca^{2+}/Mg^{2+} were added carefully to wash the cells and afterwards it was aspirated. Then 500 µl Trypsin/EDTA were added to detach the cells from the bottom of the flask. This process took approximately five minutes.
Thereafter the cells were resuspended with 4.5 ml DMEM containing 10% FBS and 1% pen-strep. The desired amount of cell suspension was transferred into a new flask which contained the appropriate amount of the growth medium.

This new flask was incubated at 37 °C and 5% CO_2.
Cell seeding was carried out when cells were needed for transfection and also for patch-clamp. For these experiments 35 mm culture dishes with single cells were essential. That is why only a small amount of the cell suspension (around 90 µl from the used flask) was transferred to each culture dish and 2 ml of the medium were added.
The culture dishes with the cells were stored in the incubator over night before transfection was made.

3.1.2. Freezing cells

A reason for freezing cells is for example to assure a steady use of the cells.
The cells were detached from the culture flask, as described under “Splitting and seeding cells“. After that the cell suspension was centrifuged in a centrifugal tube at 3000 rpm for five minutes. The supernatant was descarded if a cell pellet was seen.
This pellet was resuspended in DMEM containing 10% FBS and 1% pen-strep. 750 µl of this suspension were transferred into a 2 ml cryovial. The same amount of precooled freezing medium which contains of 50% DMEM without FBS, 30% FBS and 20% DMSO was added to the same cryovial. The vials were cooled on ice for 15 minutes. Then they were stored in a freezing box which is filled with isopropanol at -80 °C to cool down the vials slowly.
After a week one of these cryovials was thawed to check if the cells survived the freezing procedure. All the other vials were stored at the liquid nitrogen tank.

3.1.3. Thawing cells

A cryovial was transported on ice from the liquid nitrogen freezer to the lab. The vial was put into a pre-warmed 37 °C waterbath without submerging the cap.
After cleaning the vial with 70% ethanol it was transferred into the laminar air flow. Then the thawed cell suspension was given into a 15 ml tube and 8 ml DMEM containing 10% FBS and 1% pen-strep were added. After centrifugation for 5 minutes the supernatant was aspirated, the cell pellet was resuspended in 1 ml growth medium and transferred into a T25 flask with 4 ml of the media.
The cells were incubated at 37 °C, 5% CO_2 after they were surveyed under a microscope if they are alive.

3.1.4 Cell culture media and reagents

DMEM	Dulbecco`s modified Eagle medium/F12 nutrient mixture Ham [+] L-Glutamine; +4 °C Gibco by Life Technologies
FBS	Fetal Bovine Serum; +4 °C Sigma-Aldrich GmbH
PBS	Phosphate buffered saline; +4 °C Gibco by Life Technologies
PBS without Ca^{2+}/Mg^{2+} contains:	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4 pH adjusted to 7.4
Penicillin-Streptomycin	-20 °C Sigma-Aldrich GmbH
Trypsin/EDTA 0.05%	+4 °C Gibco by Life Technologies
Growth medium contains	DMEM 10% FBS 1% Penicillin-Streptomycin

3.2. Molecular biology

cDNAs of α, β and γ hENaC (wt) were a kind gift from Dr. Peter M. Snyder (University of Iowa, Carver College of Medicine, Iowa City, IA, USA).
cDNA of δ hENaC (wt) was a kind gift from Dr. Mike Althaus (Justus-Liebig-Universität, Gießen, Germany).
Enhanced green fluorescent protein (eGFP) was received from Invitrogen (Life Technologies, LifeTech Austria).

3.2.1. Transformation

DH5α cells were thawed on ice. 50 µl of these cells were aliquoted into a 1.5 ml microcentrifuge tube. Unused cells were refreezed. Then 1 µl of DNA was added to the thawed cells, carefully mixed and incubated on ice for 30 minutes.
After this time the cell suspension was heat shocked for 20 seconds in a pre-warmed 42 °C waterbath following storing on ice for two minutes.
Then 950 µl of pre-heated SOC-medium were added to the tube. This cell suspension was incubated at 37 °C and 250 rpm for one hour.
Subsequently 200 µl of the suspension were spread on a LB agar plate which contained 100 µg/ml ampicillin. So only cells containing the appropriate antibiotic resistance DNA were able to grow.
The agar plates were incubated upside down in a bacteria incubator at 37 °C and 5% CO_2 for nearly 16 hours (over night).

3.2.2. Picking colonies

The following day many cell colonies should be observable.
Single colonies were picked with a pipette tip and placed in glass tubes which each contained 3 ml LB medium and the appropriate antibiotic (100 µg/ml ampicillin).
The glass tubes were incubated at 37 °C and 250 rpm for 16 hours (over night).

All work with bacteria was performed semi-sterile under a Bunsen burner flame.

3.2.3. DNA-extraction

The DNA-extraction from Escherichia coli cells was carried out with the GeneJET Plasmid Miniprep Kit.
All steps were performed at room temperature.

2 ml of the content of each glass tube containing the grown colonies were transferred into a microcentrifuge tube and centrifuged at 1200 xg for two minutes. The supernatant was discarded.

Then each cell pellet was resuspended with 250 µl of the resuspension solution, lysed with 250 µl of the lysis solution and neutralized with 350 µl of the neutralization solution. After centrifugation for 5 minutes the supernatant was transferred to the spin column. First the columns were centrifuged for one minute and the flow-through was discarded. Then the columns were washed with 500 µl of the washing solution for two times. After centrifugation and discarding the flow-through the columns were transferred into new microcentrifuge tube, 20 µl of the elution buffer were added carefully to the center of each filter, incubated for two minutes and centrifuged. Finally the flow-through was gathered and measured with a NanoDrop Spectrophometer.
Supplementary a glycerol stock of the remaining LB medium with the cells was made.
900 µl of this cell solution were added to 900 µl glycerol, mixed well by inverting and frozen at -80 °C.
Glycerol stocks were used to pick colonies with pipette tips if this DNA is needed next time.

3.2.4. DNA-transfection

Transfection was performed with X-tremeGENE HP DNA Transfection Reagent.

This reagent, serum-free DMEM and the DNA were pre-warmed at room temperature before starting the transfection. Also the cells which were seeded into the 35 mm dishes the day before were surveyed under a microscope to check their morphology, the confluence and possible contamination.
All steps of transfection were performed under aseptic conditions. Round-bottom-glass-tubes were used.
At first 70 µl of serum-free DMEM was transferred in a labelled tube. After that 500 ng of the cDNA for the single desired wt hEnaC subunit and 150 µl eGFP were added to the tube. eGFP was necessary to control the efficiency of the transfection under a fluorescence microscope two days later.
At last 4 µl of X-treme GENE were given to the solution and incubated at room temperature for nearly 20 minutes.
In the meantime the prepared culture dish was labelled and the media was aspirated. After that the cells were washed carefully with 1 ml PBS without Ca^{2+}/Mg^{2+}.
After the incubation time the whole transfection solution was spread dropwise directly on the cells in the culture dish. Finally 2 ml growth media were added. The transfected cells were incubated at 37 °C, 5% CO_2 for 48 hours.
These transfections were only transient and so there was no expression observable after approximately four days.

3.2.5. Media and reagents

DH5α Competent Cells		-80 °C Invitrogen by Life Technologies
GeneJET Plasmid Miniprep Kit		Thermo Scientific
X-tremeGene HP DNA Transfection reagent		-20 °C Roche Diagnostics GmbH, Mannheim, Germany
Ampicilline		-20 °C Sigma-Aldrich GmbH
Glycerol		Sigma-Aldrich GmbH
LB agar		+4 °C
	contains:	20 g LB broth 15 g agar filled with ddH_2O to 1 l, autoclaved
LB medium		+4 °C
	contains:	20 g LB broth filled with ddH_2O to 1 l, autoclaved
LB broth	contains:	10 g/L tryptone 5 g/L yeast extract 5 g/L NaCl
SOC medium		+4 °C
	contains:	2% tryptone 0.5% yeast extract 10 mM NaCl 2.5mM KCl filled with ddH_2O to 1 l, pH adjusted to 7.4 with NaOH, autoclaved following filter-sterilized supplements were added: 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose

All chemicals and reagents for the media were provided by Sigma-Aldrich GmbH.

3.3. Electrophysiology

The patch-clamp experiments were carried out with transiently transfected HEK cells at room temperature 24 to 72 hours after transfection.
The cells expressed a single wt subunit of hENaC (α, β, γ or δ) and eGFP.
Electrophysiology measurements were performed with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA, USA). Data recording, storage and analysis were processed directly by a PC which was provided with pCLAMP 10.2 software (Axon Instruments, Union City, CA, USA).

3.3.1. Patch-clamp technique and whole-cell patch-clamp

First the dishes with the transfected cells were surveyed under a microscope and also a fluorescence microscope if the cells are healthy, have a high transfection efficiency and if there are well distributed single cells.
After that the medium was discarded and 2 ml of the bath solution were added.
The dish was attached on the stage of an invert microscope and the reference electrode was submerged in the extracellular solution and fixed. A fire polished glass pipette with a resistance value of approximately 5 MΩ served as a microelectrode and was filled with the intracellular solution. It was connected to the pipette holder which is linked to a micromanipulator.
The glass pipettes were made of thin-walled borosilicate glass capillaries (Harvard Apparatus, Holliston, MA, USA). These pipettes were pulled and heat polished by a DMZ Universal Puller (Zeitz Instruments, Martinsried, Germany).
An appropriate single cell was chosen under the microscope and the glass pipette tip was moved to the surface of this cell. The “gigaohm seal“ (GΩ-seal) was reached by the appliance of a light suction.
For whole-cell configuration the cell membrane had to be ruptured. This was realised by a pulsed suction or a short voltage pulse. So the whole current through all ion channels of one cell was able to measure.
After reaching the GΩ-seal an equilibration period of some minutes was applied to the cell. In this time the holding potential E_h was set to zero.
After equilibration the recordings were taken at E_h between -100 mV and +100 mV in 20 mV increments or at an E_h of -100 mV. Subsequently control recordings were taken. Then aliquots of the stock solution of TNF-α were cumulatively added directly into the extracellular solution so that the desired concentrations from 2.5 nM to 80 nM were reached.
During the recordings the GΩ-seals were regularly controlled avoiding a deficient voltage clamp.
Amiloride up to 100 µM was added at the end of the experiment to check if the measured current is the amiloride-sensitive sodium current.

3.3.2. Data-analysis

The data represent the mean ± standard error (S.E.).

Experiments were carried out on four to nine batches of independently transfected heterologous cells.

Current-voltage curves and dose-response curves as well as diagrams were plotted and the values of the half maximal effective concentration (EC_{50}) were calculated for the currents which were recorded at an E_h of -100 mV with Origin7.0 (OriginLab, Northampton, MA, USA).

3.3.3. Chemicals and solutions

Amiloride hydrochloride hydrate	Sigma-Aldrich GmbH
TNF-α	Sigma-Aldrich GmbH

Extracellular solution (bath solution):	145 mM NaCl
	2.7 mM KCl
	2 mM $MgCl_2$
	1.8 mM $CaCl_2$
	5.5 mM glucose
	10 mM HEPES
	pH adjusted to 7.4 with 1 M NaOH

Intracellular solution (pipette solution):	135 mM potassium methane sulphonate
	10 mM KCl
	6 mM NaCl
	1 mM Mg_2ATP
	2 mM Na_3ATP
	10 mM HEPES
	0.5 mM EGTA
	pH adjusted to 7.2 with 1 M KOH

All chemicals for the extracellular and intracellular solution were provided by Sigma-Aldrich GmbH.

4. Discussion

The single forms of the wt subunits of ENaC (α, β, γ or δ) were transfected transiently into HEK-293 cells.
The recordings of the whole-cell current were taken at E_h between -100 mV and +100 mV with 20 mV increments to analyse the correlation between current and voltage (I-V-graph).
Furthermore the inward current was measured as control, after adding TNF-α as well as amiloride at an E_h of -100 mV. These currents were diagrammed by bar plots.
The EC_{50}-values were also calculated from the currents at the E_h of -100 mV and plotted.
In A549 cells the EC_{50} of TNF-α was around 8.2 nM (Hazemi et al. 2010). So concentrations of TNF-α up to 40 nM respectively 80 nM were used to see if there is any effect on the current of an ENaC-subunit and to reach the steady-state.
So aliquots of the stock solution of TNF-α were cumulatively added directly into the extracellular solution. After each adding approximately two minutes were waited. Thereby a balance was able to adjust and so the influence of TNF-α was visible and recorded.
Amiloride was added at the end of the experiment to check if the increased current was due to amiloride-sensitive sodium current. So the specifity of TNF-α on this current was proved.

All declared values are the whole-cell currents.

Previous studies showed that the expression of the α-subunit of ENaC alone or in combination with the β- or γ-subunit suffices to induce a conducting channel (Canessa et al. 1994, Snyder et al. 1994). In the same way the δ-subunit of ENaC alone or in combination can form efficient channels (Kellenberger et al. 2002).
Contrary to this, the expression of the β- as well as the γ-subunit was not able to generate a functional channel respectively significant current (Canessa et al. 1994, Snyder et al. 1994).

4.1. Effect of TNF-α on α-ENaC

HEK cells were transiently transfected with the α-subunit of ENaC. The whole-cell current voltage relation was plotted for control and treatment with different concentrations of TNF-α (5 nM, 10 nM). The recordings of the sodium current were taken at E_h between -100 mV and +100 mV in 20 mV steps (Figure 5, Table 2).

As can be seen in Figure 5 and 6 as well as Table 2 the whole-cell current enhanced after adding 5 nM and 10 nM TNF-α. Thereby the increase of the inward sodium current (negative current) was greater than the outward sodium current (positive current).

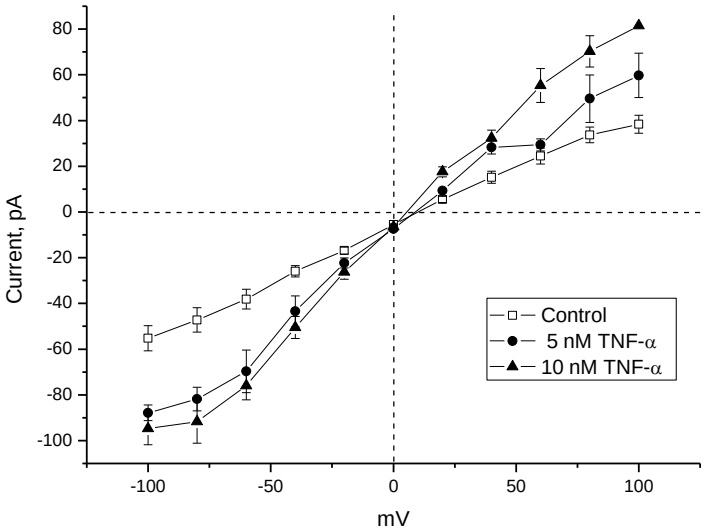

Figure 5: Whole-cell current voltage relation of α-ENaC, control, 5 nM and 10 nM TNF-α
(n = 4-13)

mV	Control (pA)	5 nM TNF-α (pA)	10 nM TNF-α (pA)
-100	-55.3 ± 5,5	-87.8 ± 3.5**	-94.8 ± 7.0**
-80	-47.2 ± 5.3	-81.8 ± 5.1**	-91.7 ± 9.4***
-60	-38.1 ± 4.3	-69.7 ± 9.3***	-76.0 ± 6.2***
-40	-25.9 ± 2.4	-43.5 ± 6.8**	-50.5 ± 4.8***
-20	-16.9 ± 1.5	-22.3 ± 2.2	-26.2 ± 3.2**
0	-5.5 ± 0.3	-7.4 ± 0.8*	-6.4 ± 1.2
20	5.5 ± 1.3	9.3 ± 0.6	17.6 ± 2.2***
40	15.2 ± 2.7	28.2 ± 2.8*	32.3 ± 3.5**
60	24.4 ± 3.5	29.4 ± 2.7	55.3 ± 7.4***
80	33.7 ± 3.4	49.5 ± 10.3*	70.2 ± 6.8***
100	38.4 ± 3.9	59.7 ± 9.7*	81.5

Table 1: Values of whole-cell current voltage relation of α-ENaC, control, 5 nM and 10 nM TNF-α */**/*** significant differences in comparison to control were determined by unpaired, two-tailed t-test (* p<0.1, ** p<0.01, *** p<0.001)

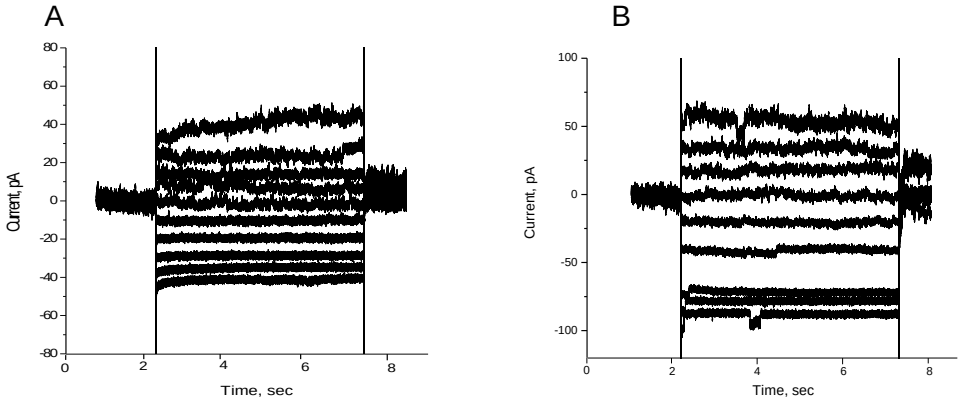

Figure 6: Original recordings from HEK-293 cells, transiently transfected with α-ENaC, in the whole-cell-mode of the patch-clamp technique
E_h between -100 mV and +100 mV in 20 mV increments, (A) control (B) 40 nM TNF-α

Most recordings were taken at E_h of -100 mV. After a control phase TNF-α was added cumulatively to the bathing solution to reach a final concentration of 40 nM. The mean values of the measured currents ± S.E. were diagrammed by bar plots (Figure 7). The whole-cell inward sodium current increased with TNF-α. The steady-state was reached at a concentration of 20 nM TNF-α.

The EC_{50}-values were also calculated from these currents at the E_h of -100 mV and plotted (Figure 8).

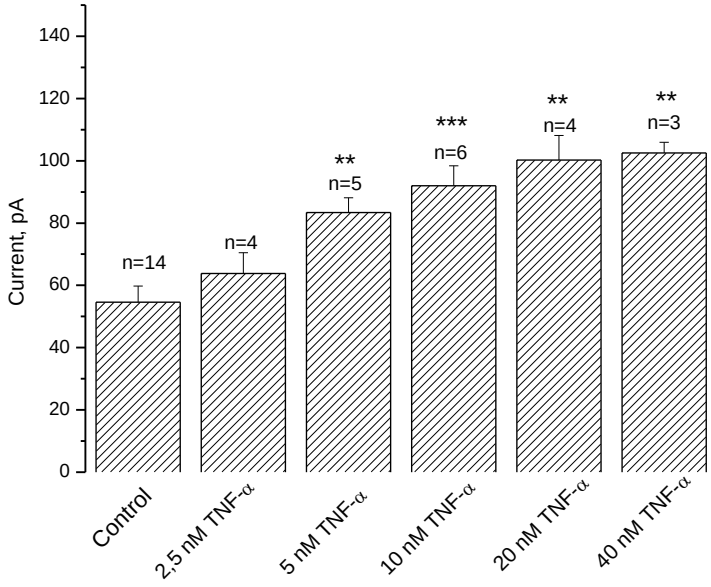

Figure 7: Effect of TNF-α on α-ENaC

/* significant differences in comparison to control were determined by unpaired, two-tailed t-test (** p<0.01, *** p<0.001)

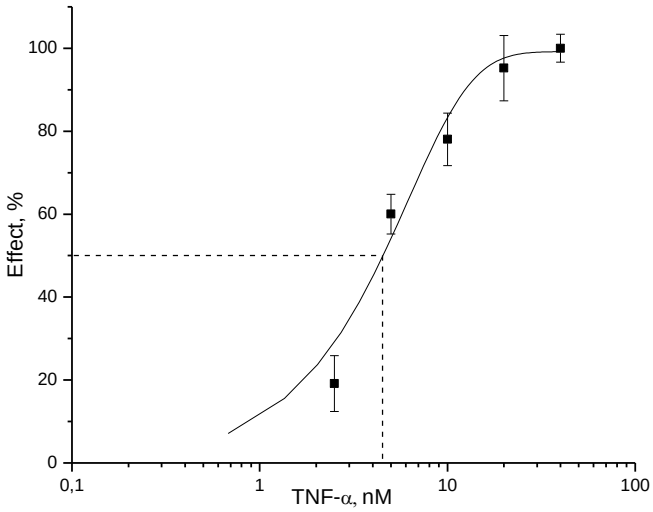

Figure 8: TNF-α dose-response-curve of α-ENaC

As shown in Figure 8 TNF-α enhances the sodium current with an EC_{50} of 4.2 nM.

4.2. Effect of TNF-α on δ-ENaC

Recordings were taken at E_h of -100 mV. After a control phase TNF-α was added cumulatively to the bathing solution to reach a final concentration of 40 nM. The mean values of the measured currents ± S.E. were diagrammed (Figure 9). The whole-cell inward sodium current increased with TNF-α concentration-dependently. As α-ENaC the steady-state was reached at a concentration of 20 nM TNF-α. At the end of the experiment amiloride was added to check if the increased current was due to amiloride-sensitive sodium current.

The EC_{50}-values were calculated and plotted (Figure 10).

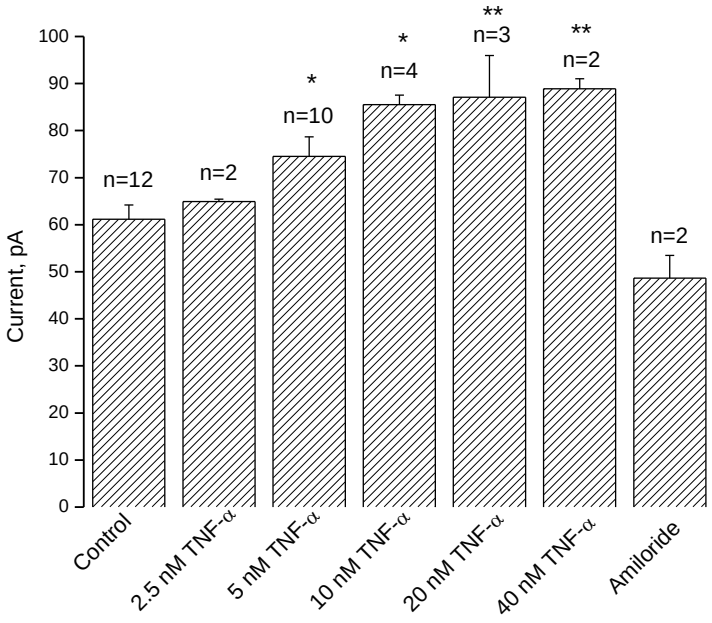

Figure 9: Effect of TNF-α on δ-ENaC

*/** significant differences in comparison to control were determined by unpaired, two-tailed t-test (* p<0.1, ** p<0.01)

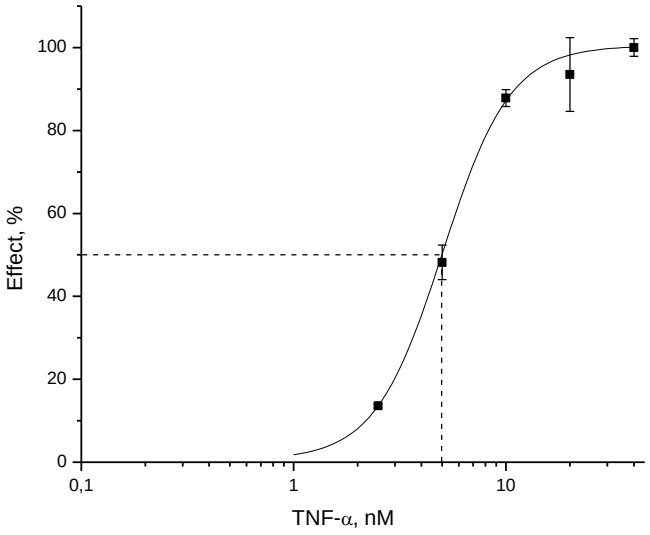

Figure 10: TNF-α dose-response-curve of δ-ENaC

The EC_{50} of δ-ENaC is about 5 nM and so similar to the EC_{50} of α-ENaC.

4.3. Effect of TNF-α on β-ENaC

Recordings were taken at E_h of -100 mV. After a control phase TNF-α was added. Here concentrations up to 80 nM were used because there was a rise of the current after adding 40 nM TNF-α. The current did not increase more at a final concentration of 80 nM TNF-α (Figure 11). The mean values of the measured currents ± S.E. were diagrammed. At the end of the experiment amiloride was added.

The EC_{50}-values were calculated and plotted (Figure 12).

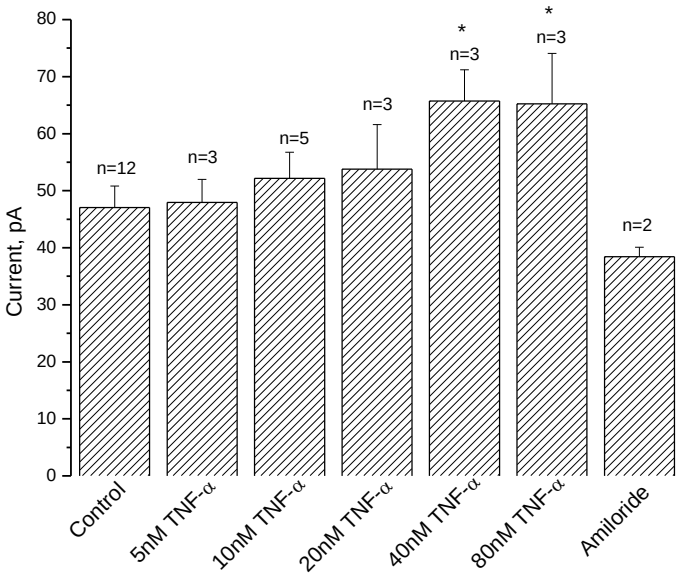

Figure 11: Effect of TNF-α on β -ENaC

* significant differences in comparison to control were determined by unpaired, two-tailed t-test (* p<0.1)

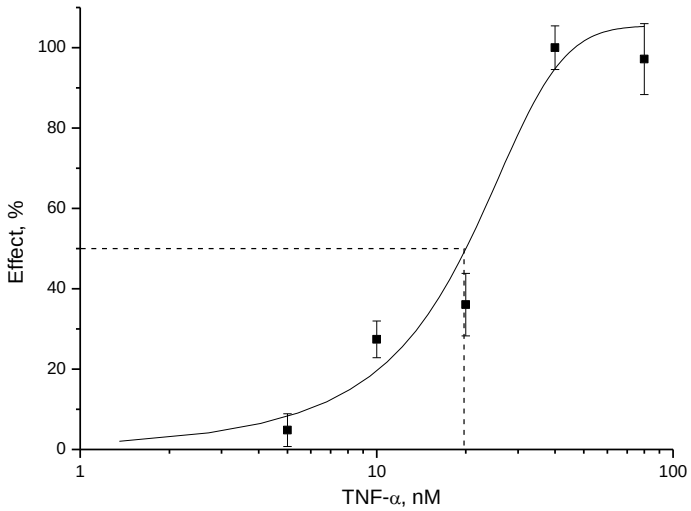

Figure 10: TNF-α dose-response-curve of β-ENaC

The EC_{50} is determined with a value of nearly 19 nM. That is four to five times higher than in the α- and δ-subunit.

4.4. Effect of TNF-α on γ-ENaC

Recordings were taken at E_h of -100 mV. After a control phase TNF-α was added. Concentrations up to 40 nM were used. But the current did not increase significantly (Figure 13). The mean values of the measured currents ± S.E. were diagrammed. At the end of the experiment amiloride was added.

A dose-response-curve was not determined.

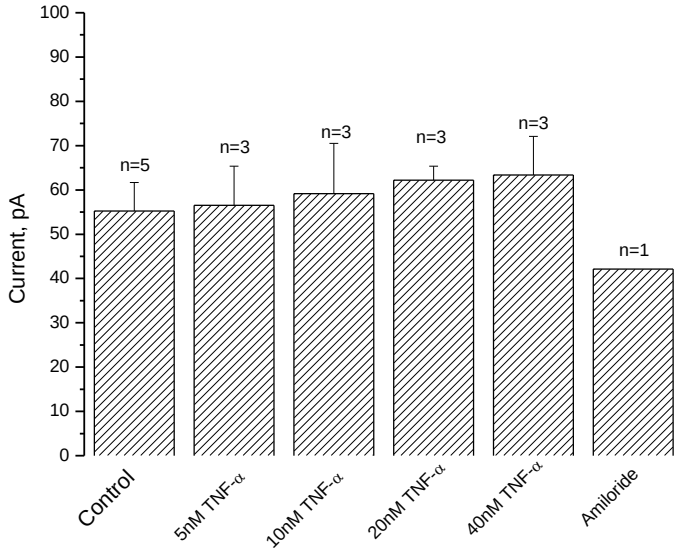

Figure 13: Effect of TNF-α on γ-ENaC

4.5. Summary

The inward sodium current was measured in all four single subunits as control, after adding TNF-α as well as amiloride at an E_h of -100 mV. Within two to three minutes after adding TNF-α a response was obtained and a few minutes later a steady-state was reached. The activation of ENaC respectively the increase of the current was reversible. The maximum effect was reached with 40 nM (α- and δ-ENaC) or 80 nM TNF-α (β-ENaC).

The EC_{50}-values of the different subunits were compared with each other in a dose-response-curve (Figure 14).

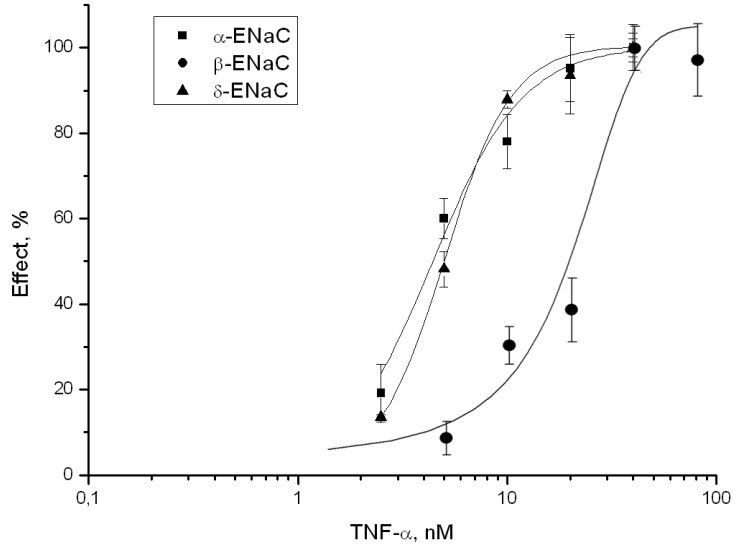

Figure 14: TNF-α dose-response-curve of α-, β- and δ-ENaC

The dose-response-curves and the EC_{50}-values of the α- and δ-subunit are nearly the same. In contrast to this the curve of the β-subunit is shifted to the right. This means that a higher concentration of TNF-α has to be added to reach the same effect. So the EC_{50} of β-ENaC is four to five times higher than in the α- or δ- subunit (Table 2). Furthermore there was no increase of the sodium current of the γ-ENaC.

hENaC (wt)	EC_{50} (nM)
α	4.2 ± 1.9
β	18.8 ± 2.9**
γ	-
δ	5.0 ± 0.28

Table 2: EC_{50} for different ENaC-subunits

** significant difference in comparison to δ-ENaC was determined by unpaired, two-tailed t-test (** $p<0.01$)

Previously it was shown that TNF-α increases the amiloride-sensitive sodium current in A549 cells which contain αβγ-ENaC with an EC_{50} 8.2 ± 0.1 nM and also δβγ-ENaC (Hazemi et al. 2010, Shabbir et al. 2013). The EC_{50}-values of the single α as well as the single δ-subunit are a little bit lower.

It was also shown that AP301 activates the amiloride-sensitive sodium current in transiently expressed HEK cells (Shabbir et al. 2013). This increase was also dependent on the appropriate subunit of ENaC. The EC_{50} was determined with 54.7 ± 2.2 nM for αβγ-ENaC. In comparison with that the effect of TNF-α is stronger than AP301 and also the induced current of TNF-α is higher (Table 3).

hENaC (wt)	TNF-α-induced current (pA)	AP301-induced current (pA)
α	48	12
β	18	3
γ	8	4
δ	27	16

Table 3: TNF-α-induced current and AP301-induced current (Shabbir et al. 2013)

So there is only less induced increase with the β- and γ-ENaC. In contrast the α- and δ-ENaC enhance more. The TNF-α induced current in α-ENaC enhances with 48 pA most, the current of the δ-ENaC increases with 27 pA. This is reversed compared to the study of Shabbir et al., where the δ-subunit induced a higher current than the α-subunit.

Therefore, the α- as well as the δ-ENaC, the pore-forming subunits, are probably necessary to form efficient channels and for an optimum effect. The specific binding-sites of TNF-α are maybe located in these subunits.

5. Conclusion

TNF-α enhances the amiloride-sensitive sodium current in ENaC. Thereby the extent of this increase depends on the particular subunit.

So there is no or only less effect with the γ- or β-ENaC whereas the α- and δ-ENaC induce a significant increase in amiloride-sensitive sodium current.

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7. Appendix

7.1. Abstract

Pulmonary oedema are developed due to an imbalance of the alveolar liquid clearance. This is due to a disturbed sodium transport by the epithelial sodium channel (ENaC) which plays a basic role thereby and regulates the fluid balance of the lung.
Hence an enhanced activity of this channel is a promising therapy option.
In addition TNF-α has two opposing effects on the alveolar liquid clearance. TNF-α, particularly its lectin-like domain, is able to activate ENaC and so to improve that clearance.
It was shown that AP301, a cyclic peptide mimicking the lectin-like domain of TNF-α, increases the amiloride-sensitive sodium current in heterologous expression systems. Though this depends according to the present subunits of ENaC.
Using the patch-clamp-technique the effect of TNF-α was analysed on the α-, β-, γ- and δ-subunit of ENaC in transiently transfected HEK-293 cells.
The experiments show that TNF-α enhances the amiloride-sensitive current due to the present subunit. Thus the half maximal effective concentration (EC_{50}) of TNF-α is 4.2 nM for α-ENaC and 5 nM for δ-ENaC. In contrast the EC_{50} of β-ENaC is with 18.8 nM four to five times higher whereas no effect is determined with TNF-α at γ-ENaC.

7.2. Zusammenfassung

Lungenödeme entstehen durch ein Ungleichgewicht der alveolären Flüssigkeitsclearance. Dieses entsteht durch einen gestörten Natriumtransport durch den epithelialen Natriumkanal (ENaC), der dabei eine wesentliche Rolle spielt und Flüssigkeitshaushalt der Lunge reguliert.
Deshalb ist eine verstärkte Aktivität dieses Kanals eine aussichtsreiche Therapieoption bei Lungenödem.
Zusätzlich besitzt TNF-α einen zweiseitigen Effekt auf die alveoläre Flüssigkeitsclearance. Durch TNF-α, insbesondere seiner Lectin-ähnlichen Domäne, kann diese Clearance durch eine Stimulation des ENaCs verbessert werden.
Es wurde gezeigt, dass AP301, ein zyklisches Peptid, welches die Lectin-ähnlichen Domäne nachahmt, den Amilorid-sensitiven Stromfluss in heterologen Expressionssystemen erhöht. Dies geschieht allerdings in Abhängigkeit der vorhandenen Untereinheiten von ENaC.
Mittels der Patch-Clamp Technik wurde der Einfluss von TNF-α auf die α-, β-, γ- sowie δ-Untereinheit von ENaC in transient transfektierten HEK-293 Zellen untersucht.
Die Versuche zeigen, dass TNF-α den Amilorid-sensitiven Stromfluss in Abhängigkeit der vorhandenen Untereinheit erhöht. So liegt die mittlere effektive Konzentration (EC_{50}) von TNF-α bei der α-Untereinheit des ENaCs bei 4,2 nM und die der δ-Untereinheit bei 5 nM. Bei der β-Untereinheit dagegen ist die EC_{50} mit 18,8 nM vier- bis fünfmal höher, während bei der γ-Untereinheit mit TNF-α keine Wirkung festgestellt werden konnte.

7.3. Curriculum Vitae

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Date and place of birth:	06.10.1985 in Karl-Marx-Stadt, Germany
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Education

Oct 2009 – ongoing	Study of Pharmacy, University of Vienna
Oct 2008 – Sep 2009	Study of Biomathematics, Ernst-Moritz-Arndt-University Greifswald
Apr 2005 – Sep 2008	Study of Pharmacy, Ernst-Moritz-Arndt-University Greifswald
1996 – 2004	A-Level at Karl-Schmidt-Rottluff-Gymnasium Chemnitz

Relevant work experience

during semester breaks 2010-2015	Samariter-Apotheke Chemnitz
Aug 2013 – Sep 2013	internship at hospital pharmacy, Klinikum Chemnitz gGmbH

Language and IT skills

German	mother tongue
English	conversational ability
French	basic knowledge
Latin	intermediate Latin certificate
IT	MS Office Programs (Word, Excel, Powerpoint)