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DIPLOMARBEIT

Titel der Diplomarbeit

"Modulation of AP301 and its target specifity on the epithelial sodium channel (ENaC) in different cell systems"

verfasst von

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Aim of the diploma thesis

Pulmonary permeability edema represents a frequent complication in a number of life-threatening lung conditions, but apart from ventilation strategies, no specific therapy yet exists for its treatment (Hazemi 2011, Tzotzos et al. 2013, Ware et al. 2001). Research studies of epithelial fluid transport by the distal epithelium in the lung have provided important new concepts regarding the resolution of pulmonary edema (Matthay et al. 2002). There is convincing evidence that active sodium transporters are responsible for the ability of the lung to remove alveolar liquid when pathological conditions lead to the development of pulmonary edema (Matthay et al. 2002).

Since the epithelial sodium channel, ENaC, in the lung is the primary site for fluid reabsorption, increasing its activity presents promising drafts regarding the treatment of pulmonary edema (Matthay et al. 2002, Yang et al. 2010). It was shown that TIP peptide AP301, which was adapted from the lectine-like domain of TNF-α and is presently undergoing clinical trials, increases the sodium current of amiloride-sensitive epithelial sodium channels in type II alveolar epithelial cells and therefore enhances liquid clearance in the lung (Tzotzos et al. 2013). However, to date the mechanism by which the TIP peptide AP301 activates ENaC-mediated sodium current remains elusive (Dulebo et al. 2013). Hence, the main aim of the diploma thesis at hand is to investigate the modulatory effect of AP301 and its target specifity on ENaC in both, the alveolar carcinoma cell line A549 as well as in heterologous cell models in order to facilitate localizing the binding site of AP301 and to understand its modulatory effect on ENaC.

Table of contents

List of figures

List of tables

List of abbreviations

A549 cells	Adenocarcinomic human alveolar basal epithelial cells
AFC	Alveolar fluid clearance
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
ASIC	Acid-sensing ion channel
AT I cells	Alveolar type 1 cells
AT II cells	Alveolar type 2 cells
CAP	Channel activating protease
CHO cells	Chinese hamster ovary cells
CNG channel	Cyclic nucleotide-gated cation channel
DNA	Desoxyribonucleic acid
ENaC	Amiloride-sensitive epithelial sodium channel
G418	Geniticin
H441	Human lung adenocarcinoma epithelial cell line
HEK cells	Human embryonic kidney 293 cells
HSC	Highly selective sodium channel
NSC	Non-selective sodium channel
LB	Lysogeny Broth
PNGnase	N-glycosidase F
RT	Room temperature
SOC	Super optimal broth with Catabolite repression
TIP	Lectin-like domain of TNF-alpha
TNF-alpha	Tumour necrosis factor-alpha

1 Introduction

1.1 Lung edema

1.1.1 Characteristics and classification

Pulmonary edema constitutes a significant life threatening disease, which is characterized by an increase in lung water content (Yang et al. 2010). The increased lung water content results from the imbalance between the rate at which fluid is passively secreted from the vascular space and the rate at which it is actively reabsorbed (Eaton et al. 2010, O´Brodovich 2005, Yang et al. 2010). Reasons for this imbalance may be left ventricular heart failure, renal insufficiency, alveolar damage or shock and lung hypersensitivity states (Yang et al. 2010). Typical symptoms of patients with pulmonary edema are, amongst others, pink-stained sputum, cough, lung-crackling sounds, anxiety, shortness of breath and other breathing difficulties (Yang et al. 2010).

Pulmonary edemas are classified in cardiogenic pulmonary edema and non-cardiogenic pulmonary edema (Yang et al. 2010). Cardiogenic pulmonary edema, which is the result of high capillary hydrostatic or venous pressure, is treated with a non-invasive ventilation for the improvement of the impaired gas exchange together with medical interventions (Yang et al. 2010). In contrast, the non-cardiogenic pulmonary edema, also called permeability edema, is a consequence of changes in the permeability of membranes due to an indirect or direct pathological process like inflammations causing pneumonia, acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) (Yang et al. 2010). Permeability edema is treated with mechanical ventilation or newer ventilation strategies like high frequency oscillatory ventilation and partial fluid ventilation (Yang et al. 2010).

Apart from those ventilation strategies no promising therapy exists for the treatment of pulmonary edema (Yang et al. 2010). Despite much research and many medical advances, pulmonary edema remains one of the more common causes for admission to hospitals and intense care units (O´Brodovich 2001).

1.1.2 Lung liquid clearance

The primary physiological purpose of pulmonary alveoli, which separate the internal vascular compartment from the air-filled compartment, is to promote gas exchange with blood (Eaton et al. 2010, Yang et al. 2010). The requirement for efficient gas exchange is having a thin liquid layer on the air facing side of the alveolar epithelium (Eaton et al. 2010). Therefore, the regulation of the amount of this luminal fluid is necessary for the proper alveolar function (Eaton et al. 2010). The reason for fluid appearing in the airways is hydrostatic pressure driving the passive movement of fluid out of the pulmonary capillaries (Eaton et al. 2010). Under normal physiological conditions, the amount of fluid which is secreted is constant since the pulmonary capillary pressure, the permeability of the epithelium and the fluid reabsorption by the alveolar epithelium are relatively constant (Eaton et al. 2010). Under pathological circumstances, the secretion may increase dramatically when either the pulmonary blood pressure or the capillary pressure is abnormally increased (Eaton et al. 2010, Yang et al. 2010). The permeability of the epithelium is enhanced by, for example, inflammation, disease or dysregulation of the expression or function of crucial ion channels (Eaton et al. 2010, Yang et al. 2010). Therefore, fluid reabsorption by the alveolar epithelium, which depends upon the rate of ion transport, is the key point for the regulation of airway fluid (Eaton et al. 2010).

The thin alveolar epithelium of the alveoli covers 99% of the large internal surface area in the lung (~100–150 m^2 in humans) and is composed of two distinct types of cells – the alveolar Type 1 (T1) and the alveolar Type 2 (T2) cells (Eaton et al. 2010, Yang et al. 2010). T1 cells are large, flat cells and cover more than 95% of the internal alveolar surface area (Eaton et al. 2010, Matthay et al. 2002, Yang et al. 2010). T2 cells are smaller, cuboidal cells which secrete pulmonary surfactant and cover only 2-5% of the internal alveolar surface area (Eaton et al. 2010, Matthay et al. 2002, Yang et al. 2010). Both, T1 and T2, cells contain ion channels, including the amiloride-sensitive epithelial sodium channel (ENaC) and therefore contribute to alveolar Na^+ absorption (Eaton et al. 2010, Matthay et al. 2002, Yang et al. 2010). As the epithelial sodium channel ENaC, especially in T2 cells, seems to be the primary site for fluid reabsorption, understanding the epithelial fluid transport induced by ENaC may

present new concepts regarding the treatment of pulmonary edema (Matthay et al. 2002, Yang et al. 2010). It was shown that the proinflammatory cytokine tumor necrosis factor TNF can promote alveolar fluid reabsoprtion *in vivo* and *in vitro* mediated by its lectin-like domain (Yang et al. 2010). Therefore, the lectin-like domain of human TNF-alpha was used as a template for the design of a series of synthetic peptides which mimic the lectin-like domain of TNF-alpha (Hazemi et al. 2010). These TIP peptides were shown to have an activating effect on amiloride-sensitive ENaC and therefore seem to present a promising treatment option for promoting the alveolar fluid clearance for patients with pulmonary edema (Hazemi et al. 2010).

1.2 The epithelial sodium channel (ENaC)

1.2.1 Function

In mammals, the epithelial sodium channel ENaC is responsible for the sodium homeostasis and is found mainly in membranes of cells in the lung, colon, kidney and sweat glands (Kashlan et al. 2011, Kellenberger et al. 2002). ENaC plays a number of diverse roles in the human body (Kashlan et al. 2011). In the lingual epithelium, for example, ENaC is responsible for the sodium specific salt taste (Kashlan et al. 2011). In the lung this channel is found in both T1 and T2 cells, where it is responsible for sodium absorption and consequently also for the lung liquid clearance (Eaton et al. 2010, Matthay et al. 2002, Yang et al. 2010).

The epithelial sodium channel is a constitutively active channel and located in the apical membrane of polarized epithelial cells (Kellenberger et al. 2002). Apart from the transport of lithium and protons, this sodium channel particularly enables the transport of sodium in cooperation with the Na-K-ATPase (Kellenberger et al. 2002, Kashlan et al. 2011). As shown in **Figure 1** ENaC mediates sodium entry into the cell and the Na-K-ATPase, which is located at the basolateral side of the cell, extrudes sodium (Kashlan et al. 2011, Kellenberger et al. 2002).

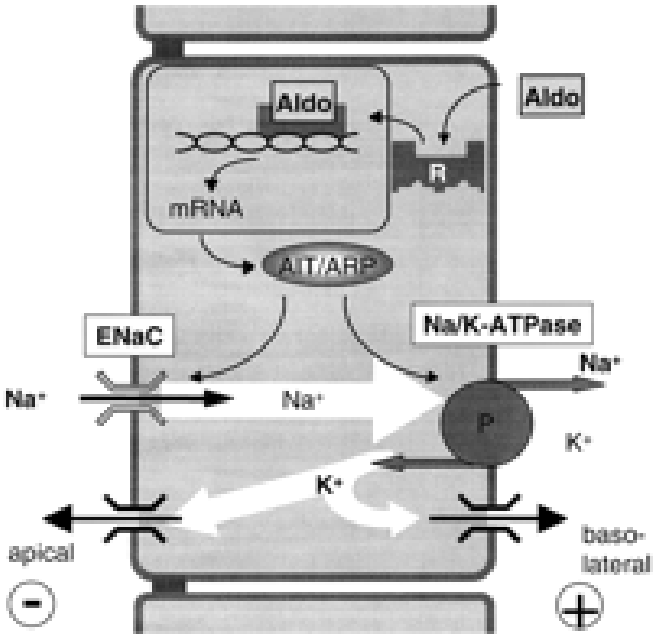

Figure 1: Transport of sodium through ENaC in conjunction with the Na-K-ATPase

At the apical membrane sodium attains the cell through ENaC. At the basolateral membrane the Na-K-ATPase is responsible for the sodium efflux.

(Kashlan et al. 2011, Kellenberger et al. 2002)

The driving force for sodium entry through ENaC is the large electrochemical gradient for sodium across the apical membrane of the cell (Kellenberger et al 2002). Since water follows sodium across the membrane due to osmolarity, ENaC is consequently responsible for the extracellular fluid volume and is hence also controlling airway surface liquid volume, mucociliary clearance and blood pressure (Enuka et al. 2011, Kashlan et al. 2011).

1.2.2 Structure

ENaC is composed of at least three homologous subunits – alpha, beta and gamma (Althaus et al. 2011, Canessa et al. 1994, Kashlan et al. 2011). The sequence analogy of these subunits amounts to 30-40%, whereupon it is supposed that the α-subunit is responsible for channel activity, whereas the β- and γ-subunits are required for channel expression and activity at the membrane (Bhalla et al. 2008, Kashlan et al. 2011, Schild et al. 2010). Each subunit consists of two transmembrane domains (TM1 and TM2), a large extracellular loop and short intracellular amino and carboxyl termini (Bhalla et al. 2008, Kashlan et al. 2011). The extracellular domain represents approximately 70% of the mass of the channel (Kashlan et al. 2011).

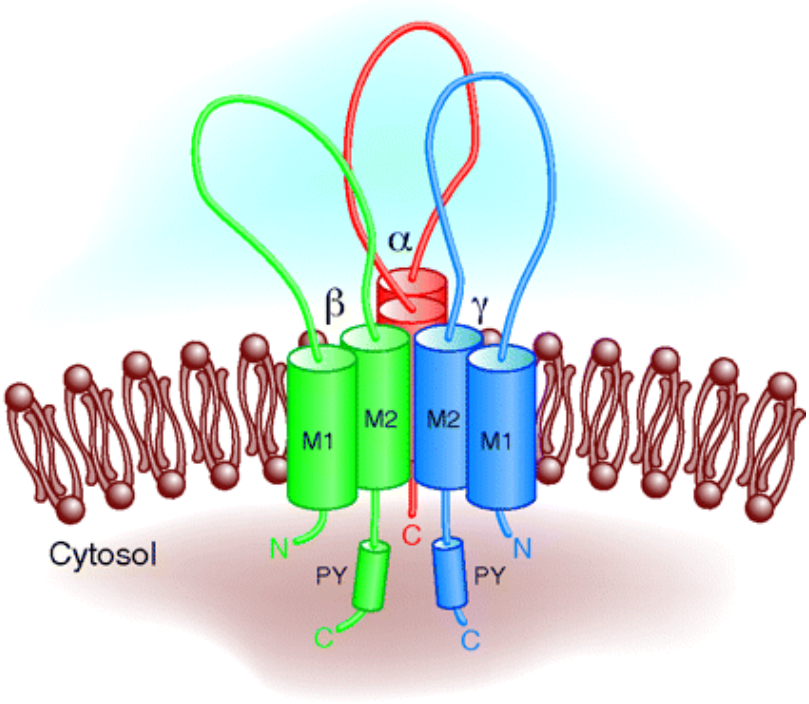

Figure 2: Structural features of the epithelial sodium channel

In the lung epithelium, ENaC may exist as a heterotrimer with a single α-, β- and γ- subunit. Each subunit has two membrane-spanning domains (M1 and M2) with intracellular N- and C-termini. The β- and γ-subunits each contain a canonical "PY" motif in their COOH-termini.

(Bhalla et al. 2008)

Furthermore, a fourth subunit of the epithelium sodium channel, termed delta subunit, was cloned in human and monkey (Ji et al. 2012). The delta-subunit seems to be closer to the alpha-subunit than to the beta- and gamma-subunit (Waldmann et al. 1995). In contrast to the alpha-subunit, the delta-subunit is found in both, nonepithelial and epithelial cells whereupon its expression seems to be associated with a numerous pathological conditions (Ji et al. 2012). It was shown that as well as alpha-, beta- and gamma-subunits also the delta-subunit is expressed in the lung (Ji et al. 2012). Despite their low homology, the delta-subunit is capable to associate just as the alpha-subunit with beta- and gamma-subunits, resulting in a functional channel (Waldmann et al. 1995). However, to what extent the delta-subunit contributes to pulmonary transepithelial sodium transport and alveolar liquid clearance remains unknown (Althaus et al. 2011).

ENaC belongs to the ENaC/degenerin ion channel family (Kashlan et al. 2011). The members of the ENaC/degenerin share only approximately 15-20% sequence identity (Kellenberger et al. 2002). Indeed they differ much regarding their functions, but in consideration of their structural characteristics, which are important for the control of the channel activity, they resemble each other (Kashlan et al. 2011, Kellenberger et al. 2002). In comparison to its family members, the epithelial sodium channel is highly sodium selective (100 Na^+ > 1 K^+), amiloride sensitive (EC_{50} of 150 nM) and has a small single channel conductance of 4-6 pS (Eaton et al. 2010, Kashlan et al. 2011). Furthermore, ENaC responds to both, chemical and external mechanical stimuli and can be blocked by submicromolar concentrations of the potassium sparing diuretic amiloride (Eaton et al. 2010, Kashlan et al. 2011, Kellenberger et al. 2002).

1.2.3 Regulation pathways

The amount of sodium which attains to the cell through ENaC depends on the one hand on the number of ENaCs located in the membrane and on the other hand on the probability of the channels to remain open (Eaton et al. 2010). The number of ENaCs in the membrane is regulated by the balance between the insertion of ENaCs into the membrane and their degradation (Eaton et al. 2010). For the process of degradation ENaC possesses a prolin rich motif PPxY on the intracellular C-Termini of its subunits (Eaton et al. 2010). Nedd4-2, an ubiquitin ligase, binds with its WW domain to this PPxY domain and causes ubiquitination of the channel (Eaton et al. 2010, Fuffieux-Daidié et al 2008). Ubiquitination is achieved by three important distinct enzyme activities. The enzyme E1 activates ubiquitin, a polypeptide which serves as a tag for the internalization (Eaton et al. 2010). Thereafter, the enzyme E2 binds to the activated ubiquitin, which is then transferred to the ubiquitin ligase E3 – in the case of ENaC E3 is termed Nedd4-2 (Eaton et al. 2010). The ubiquitin ligase Nedd4-2 interacts directly with the PPxY domain of ENaC and causes internalization of the channel (Eaton et al. 2010, Ruffieux-Daidié et al. 2008). The total number of ENaC in the membrane is relatively low compared to the number of ENaCs within the cell, what indicates that the transport of ENaC out of the endoplasmatic reticulum is an inefficient process (Eaton et al. 2010).

However, not only the number of ENaCs in the membrane is relevant for the amount of sodium which attains to the cell (Kashlan et al. 2011). The open probability, which is influenced by mechanical stress and various ligands, also plays a key role in the amount of sodium which is transported through ENaC (Kashlan et al. 2011). Generally, ENaC is a constitutively active channel, but it was revealed that its open probability varies between P=0.1 and P=0.9 and that these active ENaCs can be divided into two groups: Firstly, the ENaCs which have a high open probability as well as a long open time and secondly, those which have a very low open probability and remain closed for most of the time, also termed “near silent ENaCs” (Kellenberger et al. 2002). Furthermore, extracellular sodium is able to inhibit ENaC by a process called “self inhibition” (Kellenberger et al. 2002). However, not much is known about how ligand binding at the extracellular domain causes conformational changes in the

pore (Kashlan et al. 2011). As shown in **Figure 3** it is presumed that, on the one hand, ENaC is cleaved intracellularly by Furin and, on the other hand, ENaC is activated by cleavage of channel activating proteases called CAPs and soluble proteases like trypsin (Bhalla et al. 2008, Kashlan et al. 2011). To which extent each of ENaC's subunits is involved in cleavage and which other signaling pathways are influencing the open probability of ENaC remains a topic for future research.

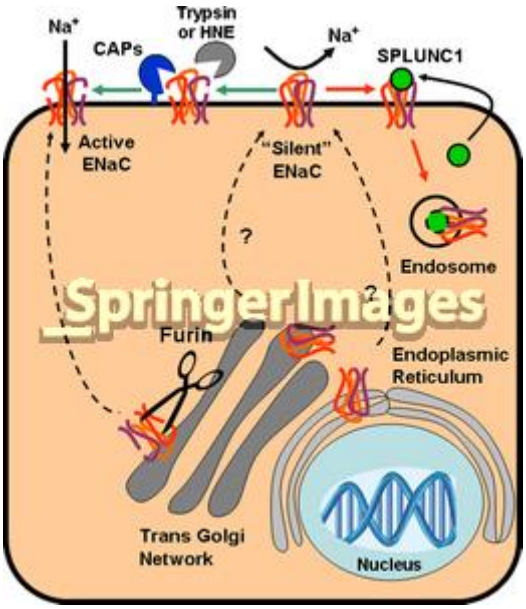

Figure 3: Different factors influence the amount of sodium which attains the cell through ENaC

On the one hand, the amount of conducted sodium depends on the insertion of ENaC in the membrane and its degradation. On the other hand, it depends on the activation which is organized by intracellular or extrecellular cleavage. Uncleaved ENaCs can be cleaved in the trans-golgi network by furin. These cleaved ENaCs are then transported to the plasma membrane where they are ready to conduct Na^+. But newly formed ENaCs are also able to bypass the furin cleavage step through an undetermined mechanism and inserted directly into the plasma membrane as uncleaved,"near silent" channels. These near silent ENaCs can then be proteolytically cleaved by cell-attached, extracellular, channel-activating proteases or by soluble proteases such as trypsin and converted into active ENaC channels.

(www.SpringerImages.com)

1.3 TIP peptide AP301 mimics the lectin-like domain of TNF-alpha

Tumour Necrosis Factor-alpha (TNF-alpha) is an inflammatory cytokine which is produced by macrophages and monocytes during acute inflammation (Idriss et al. 2000). Hence it is responsible for a diverse range of signaling events within cells leading to necrosis or apoptosis (Idriss et al. 2000). The cytokine is also relevant for the resistance against cancer and infections (Idriss et al. 2000). Since TNF-alpha is able to upregulate aleveolar fluid clearance (AFC) in pneumonia or septic peritonitis, Fukada studied the mechanisms responsible for the TNF-alpha-mediated increase in epithelial fluid transport (Fukada et al. 2001). With a group of researchers Fukada demonstrated that TNF-alpha enhances amiloride-sensitive sodium currents in alveolar epithelium and that the lectin-like domain may be responsible for this effect (Fukada et al. 2001). Therefore, the lectin-like domain of human TNF-alpha was used as a template for the design of a series of novel peptides (Hazemi et al. 2010). Several synthetic TIP peptides, which mimic the lectin-like domain of TNF-alpha, were synthesized and were shown to have an activating effect on amiloride-sensitive ENaC in A549 cells, which express ENaC endogenously (Hazemi et al. 2010, Lazrak et al. 2000).

The original TIP Peptide AP301 was designed approximately 15 years ago with the aim of mimicking the loop containing the lectin-like domain of TNF-alpha (TIP), corresponding to residues C101-E116 of wild type human TNF-alpha (TIP) (Hazemi et al. 2010, Hazemi 2011, Lucas et al. 1994). This original AP301 contains a disulphide bridge which effectively restrains the sequence of amino acid residues representing the lectin-like domain into a cyclic structure (Hazemi et al. 2010, Lucas et al. 1994). However, this disulphide bridge is an unstable element in the peptide AP301 and undesirable in a compound intended for therapeutic use (Hazemi et al. 2010, Hazemi 2011). Therefore, the aim of Hazemi's study was to explore alternative linking solutions to bring about cyclization of the linear sequence and as a consequence design a TIP peptide lacking the disulphide bridge. It should be more stable than the original TIP peptide and hence better suited for use as human medication (Hazemi et al. 2010).

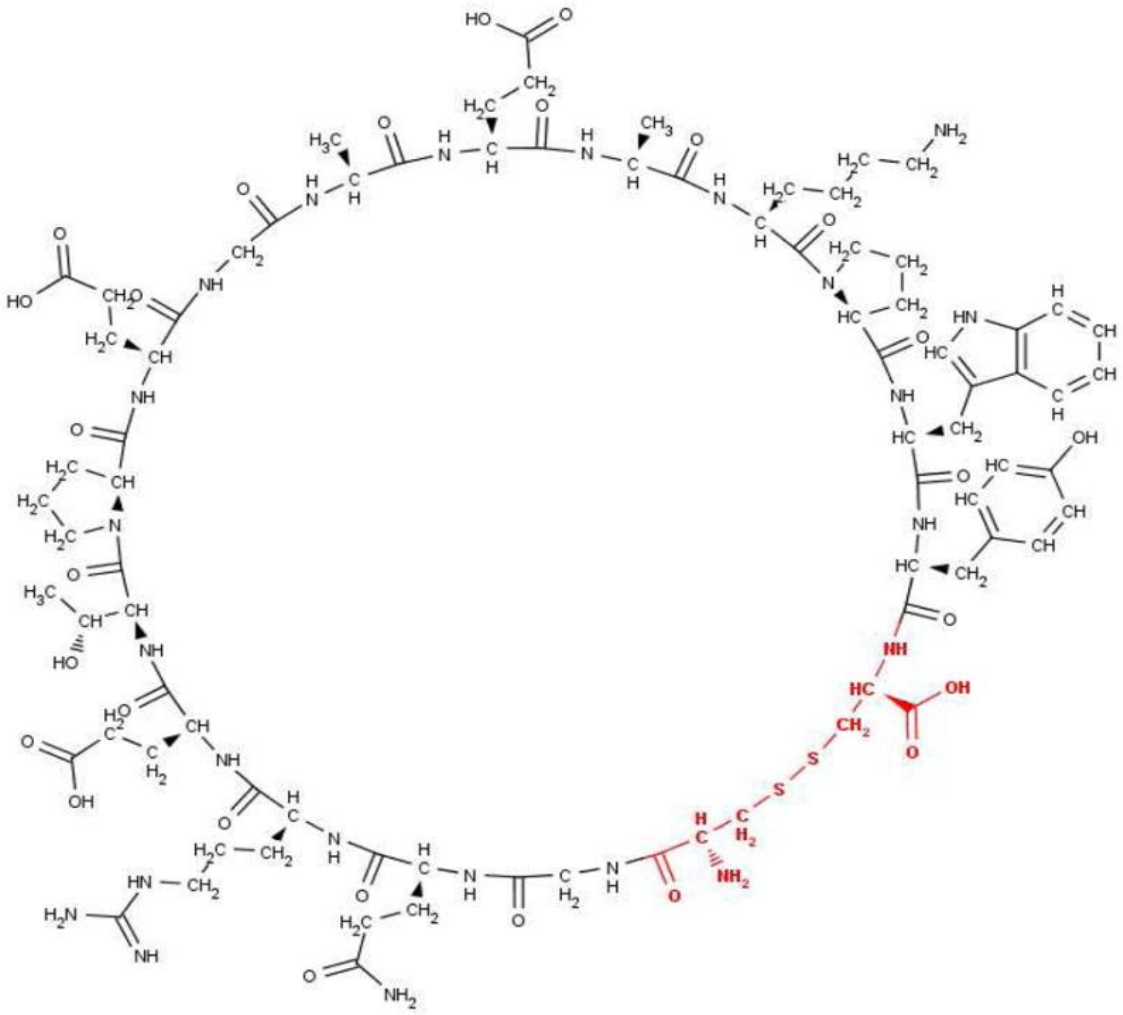

Figure 4: 2D chemical structure of the original TIP Peptide AP301 showing the disulphide bridge which effectively restrains the sequence of amino acid residues representing the lectin-like domain into a cyclic structure in red. Hazemi explored alternative linking solutions to bring about cyclization of the linear sequence in order to design a TIP peptide lacking the disulphide bridge which should be more stable than the original TIP peptide. (Hazemi et al. 2010)

Hazemi suggested that a free positively-charged N-terminal amino group on residue 1 and/or a free negatively-charged carboxyl group on residue 17 of the TIP peptide must be essential for the ENaC-activating effect (Hazemi et al. 2010). The peptides, which were designed, contained one or both of these charged groups and increased the sodium current. In contrast, TIP peptides, which did not contain these charged group, did not exert such an effect (Hazemi et al. 2010). In the novel TIP peptides cyclization was achieved mainly by formation of an amide bond (Hazemi et al. 2010).

The amino acid sequence of the TNF-alpha lectin-like domain is CQRETPEGAEAKPWYE (Hazemi et al. 2010). The identical sequence of 14 amino acids in lectin-like domains of TNF-alpha is present in the lead compound AP301 (CGQRETPEGAEAKPWYC) (Hazemi et al. 2010), which was used for the conduction of the experiments underlying this diploma thesis. AP301 is being developed as an aqueous solution (Hazemi et al. 2010). It retains its pharmacological effect after nebulisation and will be delivered to the patient by inhalation as an aerosol (Hazemi et al. 2010). Hazemi, who was part of our research group, investigated with the help of electrophysiology experiments the ability of AP301, in which cyclization was achieved by oxidation of the terminal cysteine residues to form a disulphide bridge, to enhance sodium current through ENaC (Hazemi et al. 2010). AP301 contains both, a free positively-charged N-terminal amino group on residue 1 and a free negatively-charged carboxyl group on residue 17 (Hazemi et al. 2010). The theoretical average molecular mass was 1923.1 (Hazemi et al. 2010).

2 Materials and methods

2.1 Materials

2.1.1 Cell cultures

2.1.1.1 Cell systems

CHO-K1: *ATCC* (- CCL-61), Passage: 3

HEK-293: *ATCC* (- CRL-1573), Passage: 23

A549: *ATTC* (- CCL – 185), Passage: 80

The A549 cells were kindly supplied by Prof. Walter Berger from the Medical University of Vienna. They were cultured in F12-K medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The A549 cell line – a human alveolar carcinoma cell line which overexpresses ENaC endogenously – was used for exploring the modulatory effect of AP301 on ENaC. For comparison and for assaying stable transfection, hENaC DNA was transfected into the heterologous cell systems CHO-K1 and HEK-293. HEK and CHO cells were cultured in DMEM supplemented with 10% FBS and Penicillin.

2.1.1.2 Cell culture reagents

F12K: Nutrient Mixture Kaighn's Modification, 1x nutrient mixture, [+] L-Glutamine
Gibco by Life Technologies, Life Tech Austria; +4°C

DMEM: Dulbecco's modified Eagle medium / F12 nutrient mixture Ham, [+] L-Glutamine
Gibco by Life Technologies, Life Tech Austria; +4°C

MEM: Minimum essential medium [+] Eearle's Salts, [+] L-Glutamine 1x MEM
Gibco by Life Technologies, Life Tech Austria; +4°C

FBS: Fetal Bovine Serum F0804 steril filtrated, heat inactivated

	Sigma Aldrich; +4°C
PBS:	pH 7.4 (1x) [10010015] Phosphate buffered 500ml *Gibco by Life Technologies;* +4°C
Trypsin:	25300 - 0.05% Trypsin- EDTA (1x) Phenol red *Gibco by Life Technologies;* +4°C
Penicillin:	*Sigma Aldrich;* -20°C
Geniticin:	50 mg/ml [Lot No 440790] *Gibco, Invitrogen Corporation;* -20°C
	G418 Sulphate [Lot No 108321-42-2] *Gibco by Life Technologies;* RT
Centrifuge:	Hermle Z 323K and Hermle 2 233M, HETTICH EBA 3S
Isopropanolbox	Nalgene Cryo 1°C freezing container [Cat.No.: 5100-001] *IPA*

2.1.2 Molecular biology techniques

2.1.2.1 DNA

Enhanced green fluorescent protein tagged cDNAs encoding alpha, beta and gamma human (h) ENaC were a kind gift from Dr. Deborah L. Baines (St. George's, University of London, London, UK).

Concentration of DNA used for my experiments:

Human alpha ENaC	halpha1 04.07.12 – 422 ng/µl; -20°C
Human beta ENaC	hbetaN3 21.08.12 – 360 ng/µl; -20°C
Human Gamma ENaC	hgammaC1 21.08.12 - 224 ng/µl; -20°C
$Na_{(v)}$ Beta CD8:	810 ng/µl; -20°C

2.1.2.2 Reagents

LB medium:	10 g Lysogeny broth (*Sigma Aldrich*) in 500 ml distilled H_20, autoclaved with 1 bar; +4°C
LB agar:	7.5 g Agar + 10 g Lysogeny Broth (*Sigma Aldrich*) in 500 ml distilled H_20, autoclaved with 1 bar; +4°C
SOC medium:	20 g Tryptone, 5 g Bacto Yeast extract, 0.5 g NaCl, 2.5 ml 1 M KCl with distilled H_20 to 1000 mL, autoclaved with 1 bar, completed with 0.5 ml 2 M $MgCl_2$ and 1 ml 2 M Glucose; +4°C
DH5 alpha:	*Invitrogen;* -80°C
TE Buffer:	*Fermentas*; +4°C
Miniprep Kit:	GeneJET Plasmid Miniprep Kit Pure Extreme [#K0503] *Fermentas Life Sciences;* +4°C
Cyrovials:	*Sterilin*
Kanamycin:	*Sigma Aldrich;* -20°C
Ampicillin:	*Sigma Aldrich;* -20°C
Glycerol:	*Sigma Life Science*; RT
Xtreme Gene HP:	X-treme Gene HP DNA Transfection Reagent 0,4ml enth./cont. Ethanol [Ref06366244001] *Roche;* -20°C
Dynabeads:	M2 Antiflag magnetic beads *Sigma Aldrich;* +4°C

2.1.3 Reagents for patch-clamp experiments

AP301: TIP peptide AP301 was obtained from APEPTICO Forschung und Entwicklung GMBH. *The cyclic peptide AP301 was synthesized by solid-phase peptide synthesis according to the flourenylmethyloxycarbonyl/t-butyl protection strategy on 2-chlorotitrylchloride resin by Senn Chemicals AG, Dielsdorf, Switzerland (Tzotzos et al. 2013). Cyclization in AP301 was achieved by oxidation of the terminal cysteine residues to form a disulphide bridge (Tzotzos et al. 2013). The purity of the peptide was 96,3%. m/Z (ESI) 1924.2 (M(+) +1); theoretical average molecular mass 1923.1 (Tzotzos et al. 2013).*

Amiloride: hydrochloride hydrate
Sigma Aldrich

TNF-alpha: *Sigma Aldrich*

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

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

2.2 Methods

2.2.1 Cell cultures

Working with cell cultures, included thawing the cells, changing their growth medium, splitting them- when they reached the desired confluence - and freezing them for future use. The experiments were carried out on the chinese hamster ovary cell line CHO-K1, the human epithelial kidney cell line HEK-293 and the human epithelial cancer cell line A549.

Cells were thawed from liquid nitrogen or -80°C freezer when they were needed for experiments. The cell cultures were surveyed under the microscope on a daily basis and treated appropriately to the observation. When the cells needed more growth factors, their medium was changed with new medium containing FBS. In case the cells were needed for transfections or electrophysiological experiments they were seeded in 35 mm, 60 mm or 100 mm dishes in the required amount. Whenever there was no utilization for the cell cultures after the observation, I split them mostly 1:10 when they had reached 80% of confluence. To guarantee a steady use of the cells and for not reducing their stand, the cells were sometimes re-frozen for future use.

2.2.1.1 Splitting cells

For splitting, the whole amount of the medium was aspirated from the T25 / T75 flask. Thereafter, 1.5 ml / 2 ml PBS was added to the side of the flask were no cells were growing in order to wash the remaining medium out of the flask. The flask was rinsed twice and the PBS was aspirated. Then, 500 µl - 1 ml trypsin with EDTA was added for detaching the cells from the bottom of the flask. The flask was pivoted and incubated for 5 minutes in the incubator at 37°C in a humidified atmosphere of 5% CO_2. Thereafter, the cells were resuspended with 4 - 4.5 ml appropriate growth medium and the desired amount of cell suspension was transferred into new T25 / T75 flasks containing 6 / 15 ml appropriate growth medium. The cells were maintained in an incubator at 37°C in a humidified atmosphere of 5% CO_2.

2.2.1.2 Thawing cells

Before starting with the thawing process, the water bath had to be turned on to reach temperature 37°C. 8 ml of growth medium were stored in a 10 ml centrifuge-tube on ice. After sampling out of the -80°C freezer, the vial containing the frozen cells was directly stored on ice for a few seconds and gently thawed in the water bath until the cell suspension was largely liquid, but still contained a small ice-ball. The vial was decontaminated with 70% ethanol before transferring the cells under the laminar into the centrifuge tube containing the appropriate medium. Thereafter, the cell suspension was centrifuged 5 minutes at 1000 rpm. If no pellet was to be seen, the cell suspension was centrifuged for further 2 minutes. The supernatant was aspirated and the cell pellet was resuspended with 1 ml growth medium and transferred into a T25 flask containing 6 ml of the appropriate medium. The amount of the cells was surveyed under the microscope and the cells were stored in the incubator (37°C, 5% CO_2). The cell cultures grew until reaching 70 - 90% confluence and were then either split or used for several experiments.

2.2.1.3 Freezing cells

The cells were frozen in cyrovials with 750 µl growth medium, without FBS nor DMSO, and 750 µl freezing medium, which consisted out of growth medium with 40% FBS and 20% DMSO.

First, the labeled cyrovials, the base medium (=growth medium) and the freezing medium were stored under the laminar on ice before starting with the freezing protocol. The cells were ready for freezing when having an optimal confluence of 60 - 70% in an optimal growth phase. The growth medium was aspirated from the flask and the cells were washed with 1 - 2 ml PBS. Afterwards, the cells were detached with 500 µl - 1.5 ml trypsin in the incubator (37°C, 5% CO_2) for 5 minutes. The cells were resuspended with 4 - 10 ml growth medium and centrifuged in a 15 ml centrifuge tube for 5 minutes at 1500 rpm. If a pellet was to be seen the supernatant was aspirated. The pellet was resuspended with the base medium (= growth medium) and 750 µl of the cell suspension was transferred into 2 ml cyrovials. 750 µl of freezing medium was added to each vial and the vials were stored on ice under the laminar for

15 minutes. Thereafter the cyrovials were transferred into a -80°C freezer, where they were stored in an isopropanol box for the first 2 days. The isopropanol allowed the cells to reach temperature -80°C with a rate of 1°C / minute. After a week, one of the frozen vials was thawed again for checking if the freezing process was undergone accurately.

2.2.1.4 Seeding cells

Cell seeding was performed when the cells were needed for transfections or electrophysiological experiments. For seeding cells their confluence in the T25 / T75 flasks had to reach 70 - 95%.

First of all, the growth medium was aspirated from the flask and the cells were carefully washed with 1.5 / 2 ml PBS. For detaching the cells, they were incubated for 5 minutes with 500 µl / 1.5 ml trypsin in the incubator. Thereafter, the detached cells were resuspended with 4 - 10 ml growth medium. Petri dishes with suitable sizes were labeled and 80 – 500 µl of cell suspension was transferred in 5 points to each dish. Then, 2 - 12 ml medium was added carefully directly on the cell suspension points for having a homogenous spreading of the cells on the dish surface. The cells in the dishes were stored in the incubator (37°C, 5% CO_2) for 24 - 72 hours.

2.2.2 Molecular biology

Other relevant pillars of my daily work included molecular biology techniques involving DNA extraction from the filter paper, transformation, mini prep and transfection.

2.2.2.1 DNA extraction from the filter paper

When receiving DNA, it was stored on a filter paper. The DNA on the filter paper was aseptically cut out and placed in an empty micro centrifuge-tube. 20 µl of TE buffer was added to the tube containing the filter paper. After vortexing, the sample was stored for 30 minutes at RT and was then used for the transformation into DH5-alpha cells. When the concentration of the DNA was not known, it was measured with the Nanodrop- technique.

2.2.2.2 Transformation

DH5 alpha cells, stored in -80°C freezer, were thawed on ice. 50 µl of these cells were aliquoted for each transformation into 1.5 ml micro-centrifuge tubes. Unused cells were returned into -80C° freezer. 5 µl of the DNA was added for each transformation into the tubes containing the DH5-alpha cells and were incubated on ice for 30 minutes. The DNA was then sticking to the cells. Thereafter the cell suspension was heat-shocked for 30 seconds in a 42°C water-bath and subsequent stored on ice for 2 minutes. The bacteria cells were bursting because of the heat shock what allowed the DNA to get into those cells. When cells were thereafter stored on ice, the cell membrane was shrinking again and the DNA was then placed within the DH5-alpha cells.

950 µl of pre-warmed SOC medium was added to each vial. Thereafter the tubes were incubated for 1 hour at 37°C at 225 rpm. After the incubation, the cell suspensions were centrifuged a few seconds. 200 µl of each transformation suspension was spread out on 100 mm pre-warmed (RT) selective agar plates containing agar with the appropriate resistence (for Deborah Baines´ hENaC-DNA Kanamycin selective agar plates were used). This allowed the growth of the cells which contained the antibiotic resistant DNA since the incorporated DNA had a resistance vector in its plasmid. The plates were incubated for 15 hours upside down in a bacteria incubator at 37°C and a humidified atmosphere with 5% CO_2.

2.2.2.3 Colony Picking

Glass tubes containing 3 ml LB medium and the appropriate concentration of antibiotics (0.3% Kanamycin) were prepared for picking the colonies from the agar plates after 15 hours incubation. The growth of the cells on the selective plates was rated. When there were enough single colonies, the colonies were picked with pipette tips and then transferred together with the pipette tip into glass tubes containing LB Medium, following incubation for 15 hours at 37°C and 225 rpm.

2.2.2.4 Miniprep

The Mini-Prep was carried out with the Fermentas Quick protocol and the GeneJET Plasmid Miniprep Kit. The GeneJET Plasmid Miniprep Kit was used for transferring the DNA out of the DH5 bacteria-cells into an appropriate buffer following a determination of quantification and qualification with the help of Nanodrop-technique. All following steps were carried out at RT and all centrifugations were carried out at 12000 g (~ 1100 rpm).

The growing colonies were transferred from the glass-tubes into 2 ml micro-centrifuge tubes and were centrifuged for 2 minutes. The supernatant was discarded and the cell-pellets were resuspended with 250 µl of resuspension solution. After vortexing for a few seconds, 250 µl of lysis solution was added to each tube. The tubes were inverted 5 times and 350 µl of neutralization solution was added to the cells. Thereafter, the tubes were inverted again 5 times. After the tubes were centrifuged for 5 minutes, the supernatants were loaded into GeneJet Spin columns and were centrifuged for 1 minute. Afterwards, the flow-through was discarded. The DNA was now placed on the filter and was washed with 500 µl of washing solution and thereafter centrifuged for 1 minute. The flow-through was discarded again and the washing procedure was repeated. Thereupon the micro-centrifuge tubes were centrifuged for 1 minute and the columns containing the DNA on their filters were transferred into 1.5 ml centrifuge tubes. 15 - 30 µl of Elution Buffer was carefully added to the middle of each filter with following incubation of 2 minutes. Finally after 2 minutes of centrifugation, the flow-through was collected into one tube and the DNA concentration was measured by using Nanodrop-technique.

In addition, a glycerol-stock of the remaining growing DH5-alpha colonies was prepared to reduce work for further Mini-Preps: 1 ml of LB medium containing the bacteria-cells and 1 ml of glycerol were added into a 2 ml micro-centrifuge tube. The glycerol-stock was vortexed and stored at -80°C. Next time DNA was needed, colonies were picked with pipette tips out of the glycerol stock.

2.2.2.5 DNA Transfection into cell systems

Xtreme Gene HP (stored at -20°C) was pre-warmed at RT prior to transfection. The serum-free DMEM was tested for contamination and was pre-warmed at RT. The DNA (stored in -20°C freezer) was thawed at RT and vortexed before starting with the transfection protocol. 24 hours after seeding the cells on 35 mm dishes, the cells (stored at 37°C with 5% CO_2) were first surveyed under the microscope. Cells were needed in the right growth stadium and the examination of contamination was essential. For electrophysiology experiments single colonies were needed. The amount of transfection reagents depended on the amount and confluence of the cells. For each transfection one round-bottom-glass-tube was used and labeled prior to transfection. All transfection procedures were carried out under the laminar.

75 µl of serum-free DMEM were added to the bottom of each tube. After the appropriate amount of DNA was added to the serum-free medium, the round-bottom-tubes were closed and vortexed a few seconds for achieving a homogenous DNA solution. After adding the appropriate amount of Xtreme Gene (Xtreme Gene : DNA = 1:1) the solution must not have been vortexed. Mixing the reagents was effected by shaking the tubes carefully and slowly with the fingers. The round-bottom-tubes were then stored for 30 minutes at RT. The Petri dishes containing the cells were labeled and the medium was aspirated. The cells were washed carefully with 1.5 ml PBS by adding PBS on the edge of the dish and aspirating it again as soon as possible. After 30 minutes of incubation the whole DNA solution of each round-bottom-tube was transferred to the Petri dishes by spreading it with 5 or 6 points carefully and slowly. Afterwards, 1.5 ml medium was added on the edge of the dishes. The transfected cells were incubated for 48 hours in the incubator.

2.2.3 Stable transfection

The aim of the stable transfection was to reduce the amount of work and time dedicated to it. The preparations for receiving stably transfected cells included thawing cells and achieving the optimal confluence in order to seed the cells on Petri dishes and afterwards transfecting the desired DNA into the cells. The DNA had to be extracted and its concentration was determined with the help of Nanodrop-

technique. HEK cells were used as a cell model and were cultured in DMEM supplemented with 10% FBS and Penicillin.

The cells were incubated in 100 mm dishes at 37°C/5% CO_2 and when receiving 70% of confluence transfection of the DNA was conducted. On day zero cDNA of ENaC's human alpha-, beta- and gamma subunits were transfected together with $Na_{(v)}$ Beta CD8 (Na^+ voltage gated channel) by using HP Xtreme Gene transfection reagent into the HEK cell systems. The reason for tranfecting additionally $Na_{(v)}$ Beta CD8 DNA was because Dynabeads (anti-CD8 beads), which are superparamagnetic, are able to bind to the $Na_{(v)}$ Beta CD8 and hence allow magnetic separation of the cells which successfully transfect $Na_{(v)}$ Beta CD8 and cDNA of ENaC's human alpha-, beta- and gamma-subunits and of those who do not. The transfected cells were incubated for 24 hours.

On day 1 the magnetic separation was carried out: 3 µl of Dynabeads and 8 ml DMEM were transferred into a 10 ml centrifuge tube. The medium of the 100 mm dish was aspirated and the dish was washed with 5 ml PBS. The PBS was aspirated and the medium containing the Dynabeads was transferred to the dish. After 10 minutes of incubation at 37°C the dish was pivoted under the microscope. A successful binding of the Dynabeads to the cells, which had successfully absorbed the $Na_{(v)}$ Beta CD8 DNA and hence also the ENaC DNA, was to be seen. The medium was aspirated from the dish and the dish was washed again with 5 ml PBS. After aspirating the PBS, 1 ml of trypsin was transferred to the dish following an incubation of 5 minutes. Thereafter, 3 ml medium were added to the dish, resuspended and the cell suspension was transferred into a glass tube. Instead of using the Macs Seperator system for separating the successfully transfected cells from the cells which did not contain the desired DNA we decided to use a simple magnet which was manually held to the side of the glasstube containing the cell suspension. After 2 minutes of waiting, the Dynabeads, which were still binding to the successful transfected cells, were gravitated from the magnetic field and concentrated at the edge of the glass tube because of the magnetic power. This process facilitated aspirating the rest of the cell suspension containing the cells which had not expressed the DNA and would therefore die after adding antibiotics since the DNA plasmid contained a vector with

antibiotic resistance. This would result in numerous cell deaths and as a consequence in a higher risk for contamination. The labeled cells were resuspended with 3 ml of DMEM and transferred into a T75 flask containing 12 ml DMEM supplemented with 10% FCS, 400 µg/ml G418 (Geneticin) and 1% Penicilin.

On the following days the flask was surveyed thoroughly. Depending on the growth behavior of the cells either medium change with the same or a higher concentration of G418 or cell splitting with the same or a higher concentration of G418 was conducted. Within 15 days, the concentration of G418 was increased step by step up to a concentration of 1.5 mg G418 / ml DMEM. When achieving stably transfected HEK cells, without any indication of high stress level, they were used for patch-clamp experiments in order to investigate the modulatory effect of AP301.

2.2.4 Patch-clamp technique – experimental procedure

All patch-clamp experiments were carried out with the help of Dr. Waheed Shabbir, who introduced me to the system and helped me with the results and the data.

The patch-clamp experiments were performed 24 - 48 hours after transfecting the DNA into the cell systems. Stably tranfected cells needed to be in an unstressed stadium after treatment with the desired concentration of Geneticin. When the transfected cells as well as the homologous A549 cells had 70 % of confluence and there was no contamination or cell death to be seen, they were seeded into dishes and patch-clamp experiments were carried out after 24-48 hours.

2.2.4.1 Whole-cell recording mode (Hamill et al. 1981)

The dishes containing the single colonies of cells were fixed on the stage of an Axiovert 100 microscope (400x magnification; Carl Zeiss, Germany). The chambers contained 1 ml of a bath solution of the following composition (in mM): 145 NaCl, 2.7 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5.5 glucose and 10 HEPES, titrated to pH 7.4 with 1 M NaOH solution. The cells were totally submerged throughout. Under the microscope one suitable cell was chosen. Thereafter, a fire polished glass pipette, which was used as

the microelectrode, was pushed against the surface of the cell. Micropipettes were pulled from thin-walled borosilicate glass capillaries (World Precision Instruments, Inc., FL, USA) with a Flaming Brown micropipette puller (P87, Sutter Instruments, CA, USA) and polished on a micro-forge (Narishige, Tokyo, Japan) to obtain electrode resistances ranging from 2.0 to 3.5 MΩ. The glass pipette contained a solution of the following composition (in mM): 135 potassium methane sulphonate, 10 KCl, 6 NaCl, 1 Mg_2ATP, 2 Na_3ATP, 10 HEPES and 0.5 EGTA, adjusted to pH 7.2 with 1M KOH solution. A gentle suction was applied to obtain a "gigaohm seal" between the cell membrane and the glass pipette.

For the whole-cell recordings, the membrane under the pipette tip was subsequently ruptured by application of continued suction, such that current flowing through all the ion channels in one cell could be measured. The membrane of a cell, whose interior came into contact with the pipette solution, was clamped to a defined potential via a pre-amplifier (CV-203BU Headstage, Axon Instruments) and amplifier (Axopatch 200B, Axon Instruments). At the beginning of each measurement the holding potential, E(h), was set to zero by application of the appropriate current. After equilibration, the cell membrane was clamped at a holding potential of -100 (E(h)= -100mV). Currents were filtered at 5 kHz and recorded at 10 kHz. Acquisition, storage and analysis of data were carried out with the pCLAMP 10.0 software (Axon Instruments, CA, USA).

After "gigaohm" seal formation, the voltage was clamped at -100 mV for control measurements. Thereafter, AP301, which was dissolved in distilled water, was added directly to the bath solution in the assay chamber. The solution of AP301 was of such composition that the addition of 0.5 µl AP301 to 1 ml bath solution resulted in a final concentration of 120 nM. The inward sodium current was measured following the addition of AP301. The results were analyzed using the computer program pCLAMP, version 10.0. After recording the current with AP301, amiloride (up to 1 mM) was added to the bath solution in order to prove that the increase in current affected by AP301 was indeed the one flowing through ENaC.

2.2.4.2 Inside-out and outside-out patches (Hamill et al. 1981)

With inside-out and outside-out experiments not the whole-cell current through the entire ion channels in the cell membrane is measured but single channels in the membrane patch, which is attached to the micropipette. Bath and pipette solutions had the following composition (in nM): 145 potassium methane sulphonate, 5 $MgCl_2$, 40 mannitol, 10 HEPES and 5.5 glucose, pH 7.4. The preparations for achieving inside-out or outside-out patches are similar to those required for whole-cell recordings: The glass pipette is pressed against the cell membrane; a seal has to be formed upon applying slight suction, whereby the seal between membrane and pipette increases, forming a cell-attached patch. To obtain an inside-out patch the glass pipette has to be quickly detached from the cell after gigaohm formation. Disruption of the outer vesicle membrane is done by passing the pipette tip briefly through the air-water interface of the bath. The short exposure of the pipette tip to air results in subjecting the intracellular surface of the membrane to the external solution. This technique is particularly used for exploring the effect of compounds, which are binding to the intracellular side of channels. In contrast, outside-out patches emanates from whole-cell configuration, which is achieved by applying strong suction to destroy the membrane isolated by the patch pipette. Pulling the pipette away from the cell results in exposing the extracellular side of the membrane to the external solution, what is used for studying the effect of compounds binding to the extracellular side.

3 Results and Discussion

Since the epithelial sodium channel, ENaC, especially in the AT II cells, is the primary site for fluid reabsorption, increasing its activity presents promising concepts regarding the treatment of pulmonary edema (Matthay et al. 2002, Yang et al. 2010). To date, the mechanism by which TIP peptide AP301 activates ENaC-mediated sodium current remains elusive (Dulebo et al. 2013). Hence, the main aim of this diploma thesis was to investigate the modulatory effect of AP301 and its target specifity on ENaC. The experiments were carried out in three cell lines: in the A549 cell line, a carcinoma cell line, which overexpresses ENaC endogenously and in two cell models, the CHO and the HEK cell line, which were used for exploring the effect of AP301 in heterologous expression systems. All data shown are the results of whole-cell and single-channel patch-clamp techniques, which were carried out together with Dr. Waheed Shabbir.

3.1 Effects of TNF-α and AP301 on the amiloride-sensitive sodium current in A549 cells

Since there is a huge difficulty in isolating and culturing ATII cells, ATII cell-like cell lines, such as A549 cells, are used for characterizing the properties of ion channels (Lazrak et al. 2000). A549 cells are originated from a human alveolar carcinoma cell line, possess many characteristics of alveolar type II cells and are therefore a widely accepted model for exploring ENaC electrophysiology (Lazrak et al. 2000, Lieber et al. 1976). Furthermore, it is known that A549 cells possess ENaC´s alpha-, beta- and gamma-subunits with biophysical properties similar to those of ATII cells (Lazrak et al. 2000).

It was shown that the proinflammatory cytokine tumor necrosis factor - alpha is able to promote alveolar fluid reabsoprtion *in vivo* and *in vitro* mediated by its lectin-like domain (Yang et al. 2010). Furthermore, there is great evidence that TNF-alpha is able to activate amiloride-sensitive sodium currents in A549 cells (Fukada et al.

2001). Therefore, TIP peptide AP301, which mimics the lectin-like domain of TNF-alpha, was synthesized and was shown previously in our lab in Hazemi's series of experiments to have an activating effect on ENaC in A549 cells, which express ENaC endogenously (Hazemi et al. 2010). Hazemi added amiloride, which inhibits the sodium flow through ENaC, in control experiments and hence identified the registered current as the amiloride-sensitive sodium current. To prove the specifity of the test compound on the amiloride-sensitive sodium current, amiloride was added after addition of AP301 when a steady-state effect of AP301 has been reached (Hazemi et al. 2010). Amiloride reversed the enhancing effect what proved that the increase of the current with AP301 is due to the amiloride-sensitive sodium current (Hazemi et al. 2010).

For being sure whether the A549 cells we use, behave the same way, I repeated Hazemi's experiments with our A549 cells, with the aim to demonstrate the enhancing effect of AP301 on the sodium conductance of the amiloride-sensitive epithelial sodium channel before investigating AP301's target specifity in a new series of experiments.

	Na^+ Current (pA)	**Na^+ Current with 10 µM amiloride (pA)**
Control	139 ± 4	48 ± 5
120 nM AP301	1021 ± 7	63 ± 2
10 nM TNF-α	1025 ± 9	69 ± 6

Table 1: Sodium currents with and without modulation of TNF-alpha and AP301 and their block with amiloride in A549 cells

Cells were patched in whole-cell mode. Mean values of inward currents during control phase, following addition of indicated concentrations of AP301, TNF-α and amiloride to the bath solution (n=8). Values are mean +/-SE.

The experiments were carried out with 120 nM AP301 and 10 nM TNF-alpha since Hazemi showed in her experiments that a maximal effect was achieved with these concentrations (Hazemi 2011). In her experiments, increasing the concentrations of the modulating substances did not further increase the current enhancing effect (Hazemi 2011). As shown in **Table 1,** in whole-cell patch-clamp assay the sodium

current was blocked more than 60% by using 10 µM amiloride, what identified the measured current as the amiloride-sensitive sodium current. The current was increased more than 900%, when steady state has been reached with AP301 (120 nM) and with TNF-alpha (10 nM). This AP301- and TNF-alpha-dependent increase in current was blocked also by the addition of amiloride, an indication that the enhanced current was the sodium current through ENaC. These data show that AP301, increases the sodium current also in the A549 cells we use for our experiments.

3.2 Target specifity of AP301

As shown above, there is much evidence that AP301 increases the sodium current of ENaC in A549 cells. However, to our knowledge it is not known to which extent AP301 affects other ion channels or ion pumps directly or indirectly, which contribute to the sodium homeostasis (Hazemi et al. 2010). The epithelial sodium channel is a highly selective sodium channel (HSC), what means that ENaC has a very high selectivity for sodium over potassium (100:1 to 1000:1) (Kellenberger et al 2002). The reason for this may be subjected to the pore's ability to select for sodium, partly because of its size (Kellenberger et al 2002). This idea also explains why lithium and protons, which are smaller than sodium, are able to pass the channel (Kellenberger et al 2002). Potassium, for example, which is bigger than sodium, is not able to pass the channel (Kellenberger et al 2002). Furthermore, there is much evidence that, besides ENaC, A549 cells also express cyclic nueclotide-gated ion channels which may contribute to the modulatory effect of AP301 (Karle et al. 2004, Lazrak et al. 2000, Xu et al. 1999). Since cultured AT II cells also contain CNG channels, whereupon their expression depends on culture conditions and on the duration of cultivation (Kemp et al. 2001), it is of importance to investigate the contribution of these channels to the modulatory effect of AP301.

3.2.1 Replacement of Na^+ with N-Methyl-D-Glutamine in the bath solution

To confirm that the increase of the current elicted by AP301 is the result of the modulatory effect of AP301 on the sodium current of ENaC, ion substitution experiments were performed in A549 cells (Tzotzos et al. 2013). In the first series of experiments NaCl was replaced with N-methyl-D-glucamine (NMDG)-Cl (140 mM) in the external solution of a whole-cell patch-clamp assay using A549 cells to characterize the ENaC-specific modulatory effect of AP301 (Tzotzos et al. 2013). NMDG is a large, cell impermeant, positively charged molecule, which acts like sodium but is no sodium and hence does not get conducted through ENaC (Lazrak et al. 2002). AP301 was added to a final concentration of 120 nM to the external solution since it was observed in previous experiments that a maximum response in A549 cells is achieved with this concentration of AP301 (Hazemi 2011, Tzotzos et al. 2013).

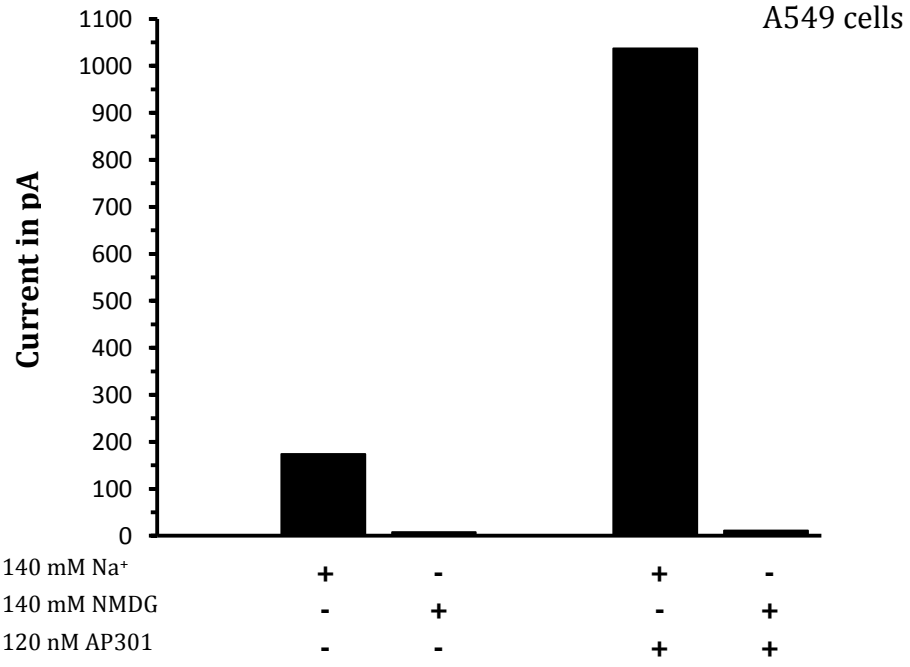

Figure 5: Ion-specific modulation of AP301 - Replacement of NaCl with NMDG-Cl

Effect on AP301-induced current of replacing NaCl in the bath solution with equimolar concentration of NMDG-Cl (140 mM) in a whole-cell patch clamp assay using A549 cells (n=5). Mean values (+/-SE) of inward currents in cells clamped at -100 mV.

(Tzotzos et al. 2013)

The first (left) bar in **Figure 5** shows the sodium current of 174 ± 9 pA with NaCl in the external solution but without NMDG nor addition of AP301. The second bar shows that there is no sodium flow through ENaC, when sodium chloride is replaced with NMDG chloride in the bath solution. The third bar presents the modulatory effect of 120 nM AP301 on ENaC with sodium but without NMDG in the external solution (1037 ± 9 pA). The fourth bar demonstrates that there is no current when AP301 is added and sodium is substituted with NMDG and hence confirms the assumption that the modulation of AP301 works choride-independent and that AP301 affects ENaC and enhances its sodium current.

3.2.2 Effect of AP301 on CNG conductance

Another model cell line which mimics alveolar type II cell characteristics is the H441 cell line (Albert et al. 2008). H441 cells are derived from human Clara cells found in the bronchiolar epithelium (Lazrak et al. 2002). Like A549 cells, H441 cells are also expressing ENaC endogenously (Albert et al. 2008). Since Albert knew, that also CNG channels have been described in lung epithelial cells, he used L-Cis Diltiazem to block CNG activity in H441 cells (Albert et al. 2008). Nonselective cyclic nucleotide-gated (CNG) cation channels open in presence of cyclic nucleotides whereby cations flow into the cell and cause depolarization (Hazemi 2011, Kemp et al. 2001). CNG channels have been shown to be expressed in ATII cells with the consideration that these channels may contribute to alveolar liquid clearing capacity (Kemp et al. 2001). In order to investigate whether these CNG channels might be involved in the current enhancing effect of AP301, whole-cell patch-clamp experiments were carried out in A549 cells using the CNG channel inhibitors Zn^{2+} and L-Cis Diltiazem (Albert et al. 2008, Kemp et al. 2001).

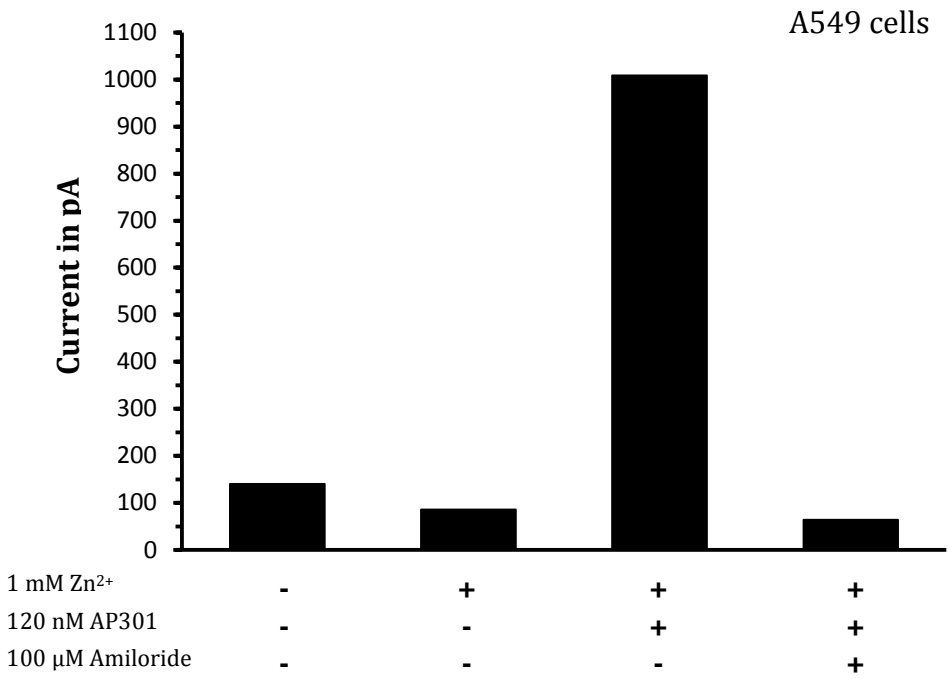

Figure 6: Effects of CNG channel inhibitor Zink on the amiloride-sensitive Na^+ current induced by AP301 in A549 cells

Cells were patched in whole-cell mode. Inward current elicited by a pulse of -100 mV. Mean values of inward currents during control phase, following sequential addition of Zn^{2+} (1 mM), AP301 (120 nM) and final addition of amiloride (100 µM) to the bath solution (n=5). Values are mean +/- SE.

(Tzotzos et al. 2013)

Addition of 1 mM Zn^{2+} to the bath solution resulted in a reduction of the current from a control value of 140 ± 3 pA to 86 ± 10 pA and the subsequent addition of AP301 (120 nM) brought about an increase in the current to 1009 ± 16 pA. A final additon of amiloride (100 µM) reduced the current to 64 ± 11 pA. Thus, CNG channels are likely to be present in A549 cells, as evidenced by the initial fall in current by ~54 pA following the addition of Zn^{2+}. However, CNG channels do not contribute to the current enhancing effect of AP301, since its effect was still observed in the presence of Zn^{2+} at concentrations found elsewhere to be inhibitory to CNG channels (Tzotzos et al. 2013).

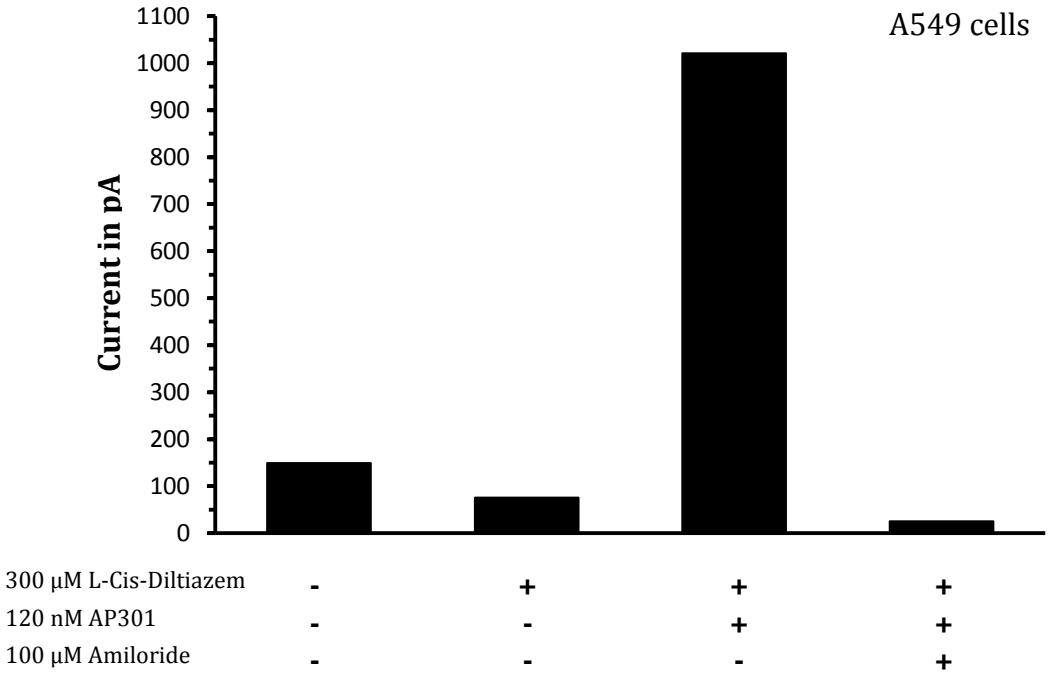

Figure 7: Effects of CNG channel inhibitor L-Cis-Diltiazem on amiloride-sensitive Na^+ current induced by AP301 in A549 cells

Cells were patched in whole-cell mode. Mean values of inward currents during control phase, following sequential addition of L-Cis-Diltiazem (300 µM), AP301 (120 nM) and final addition of amiloride (100 µM) to the bath solution (n=3).

(Tzotzos et al. 2013)

Similarly, addition of 300 µM L-Cis-Diltiazem to the bath solution resulted in an initial reduction of the current from a control value of 149 ± 4 pA to 76 ± 6 pA and subsequent addition of AP301 (120 nM) resulted in an increase in the current to 1021 ± 5 pA. The final addition of amiloride (100 µM) resulted in a reduction of the current to 25 ± 4 pA. This also reinforces the results obtained with Zn^{2+} and suggests that the current enhancing effect of AP301 is exclusively due to its effect on the amiloride-sensitive Na^+ channels (Tzotzos et al. 2013).

3.2.3 Effect of AP301 when ENaC is blocked by AICAR

Moreover, Albert used H441 Clara-like airway epithelial cells, which were cultured as monolayers, for exploring the effect of AICAR on sodium channels since H441 cells exhibit a number of similarities to human airway epithelial cells in vivo human airway (Albert et al. 2008). With cell-attached patch-clamp recordings he identified two different types of amiloride-sensitive sodium channels in the apical membrane of H441 cells: an ENaC-like high sodium selective channel (HSC) with a conduction of about 5 pS and a non-selective cation channel (NSC) with a conduction of about 18 pS. Both types of channels were responding differently to amiloride, a potent blocker of ENaC (Albert et al. 2008). Since HSCs were significantly more sensitive to inhibition by amiloride than the NSCs the sodium current through HSCs was identified as the amiloride-sensitive sodium current (Albert et al. 2008).

In his experiments it was also shown that the activator of AMP-activated protein kinase (AMPK) AICAR inhibits apical amiloride-sensitive sodium conductance in H441 monolayer cells (Albert et al. 2008). Therefore, we wanted to test its effects on ENaC in A549 cells. Our expectation from these experiments was the final affirmation that AP301 modulates the sodium current exclusively through ENaC. Our hypothesis relied on the perception that, when ENaC is blocked by AICAR, no modulatory effect of AP301 will be seen. Consequently we should be able to exclude that AP301 also affects the current of other ion channels.

Since Albert pretreated his H441 with 2 mM AICAR with the result that 90% of the channel's activity was blocked, we also pretreated our A549 cells with 2 mM AICAR prior to the patch-clamp assay (Albert et al. 2008).

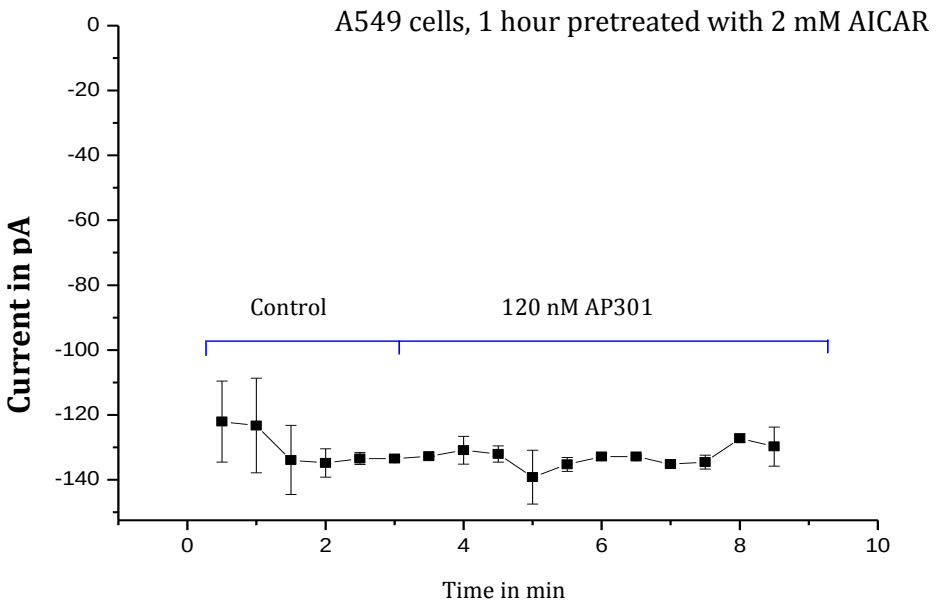

Figure 8: Pretreatment of AICAR causes loss of the modulatory effect of AP301 on ENaC in A549 cells

Cells were patched in whole-cell mode. A549 cells were 1 hour pre-incubated with 2 mM AICAR. After the control phase to establish a steady baseline current, 120 nM of AP301 was added to the bath solution and the current was measured for further 5 minutes.

Normally AP301 increases the sodium current but after 1 hour of pre-incubation with AICAR, there was absolutely no enhancing effect to be seen (Tzotzos et al. 2013).

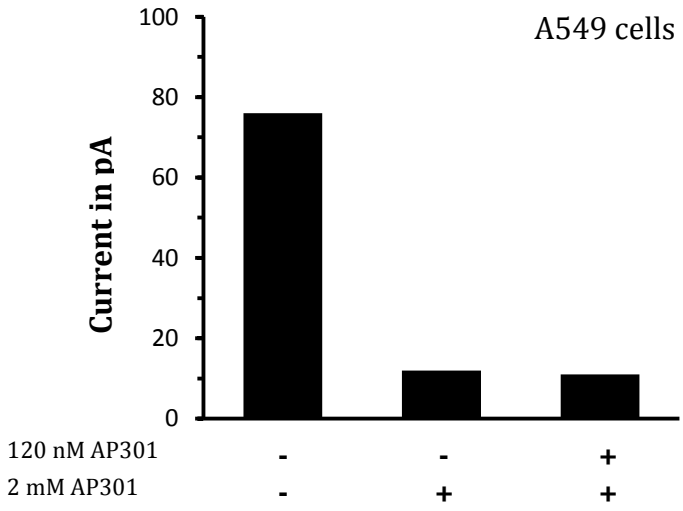

Figure 9: Pretreatment of AICAR causes loss of the modulatory effect of AP301 on ENaC in A549 cells

Cells were patched in whole-cell mode. A549 cells were 1 hour pre-incubated with 2 mM AICAR. After the control phase to establish a steady baseline current, 120 nM of AP301 was added to the bath solution and the current was measured for further 5 minutes.

Figure 9 again demonstrates that AP301 does not enhance the amiloride-sensitive sodium current when added to the external solution after ENaCs were blocked with AICAR.

The results from the ion substitution and inhibition experiments show on the one hand that the TIP peptide AP301 modulates exclusively the amiloride-sensitive epithelial sodium channel and not CNG nor chloride channels and on the other hand that AP301 interacts directly with ENaC causing a substantial increase of the amilorid-sensitive sodium current after addition to the bath solution.

3.3 Effect of AP301 on ENaC in heterologous expression systems

We have previously shown in A549 cells that AP301 enhances the sodium current through ENaC more than 900% when added to the bath solution. After a steady state has been reached, addition of amiloride resulted in a total regression of the increased current, what confirmed that the TIP peptide induced enhancement was due to the amiloride-sensitive sodium current (**Table 1**). We further sought to analyze the post-translational modification of ENaC and its possible effect on AP301 modulation in a heterologous expression system. We intended to investigate the modulatory effect of AP301 in cell models, which do not express ENaC endogenously, in order to analyze the effect of AP301 on different subunits. Therefore, we transiently and stably expressed alpha, beta and gamma hENaC DNA in HEK cells and repeated the experiments with AP301 and amiloride. To our knowledge this was the first study to show the modulatory effect of AP301 in heterologous expression systems.

3.3.1 Sodium conductance of stably transfected ENaC in HEK cells and its modulation with AP301

To facilitate the laboratory work I tried to achieve stably transfected human ENaC in HEK and CHO cells by using Geniticin (G418) for selection pressure. Since the Plasmid of ENaC contains a vector with G418 resistance, we added G418 to the cell culture's normal growth medium by starting with a concentration of 400µg/ml and ending up with a concentration of 1.5mg/ml. After several attempts, optimization of

the stable-transfection protocol was attained to obviate unnecessary stress and early death of the cells. Fundamental improvements included firstly magnet separation of cells, which expressed ENaC successfully and of cells, in which expression failed at the beginning of the experiment for preventing overgrowth. Secondly, slower enhancement of the G418 concentration resulted in less stress conditions and therefore less cell deaths. Furthermore, cell splitting and medium change were reduced to the minimal to avoid contamination. We successfully achieved CHO and HEK cells, which stably expressed ENaC's subunits alone, all subunits together and dimeric combinations, showing stability after freezing and afterwards thawing them again. Consequently, we measured the sodium current of stably tranfected alpha-, beta- and gamma-subunits of hENaC in HEK cells first without and later with modulation of AP301.

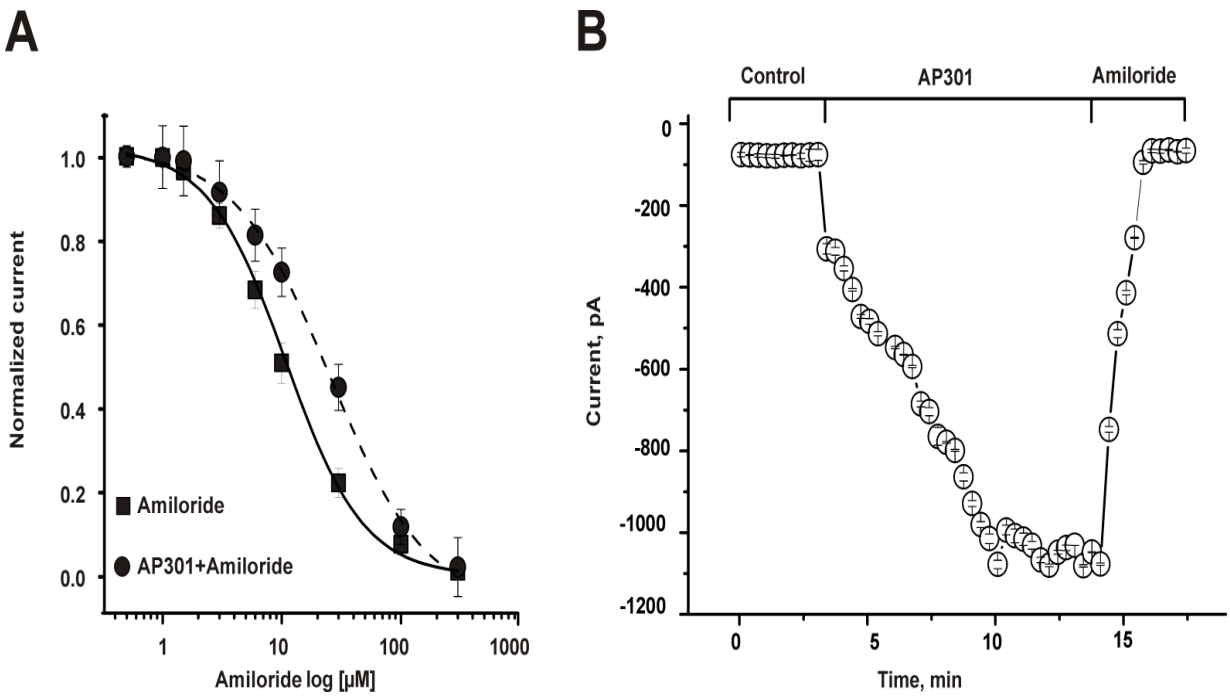

Figure 10: The modulatory effect of AP301 on ENaC in HEK cells and its block with amiloride

Dose-response experiments with amiloride in the presence and absence of AP301 and the effect of AP301 on the sodium current using stably fransfected HEK cells . Cells were patched in whole-cell mode. Mean values (+/-SE) of inward currents in cells clamped at -100 mV (n=5).

(Shabbir et al. 2013)

Figure 10 A shows dose-response experiments with amiloride in the presence and absence of AP301 in HEK cells tranfected with alpha, beta and gamma hENaC. Addition of amiloride up to 1 mM resulted in a block of ENaC current but also in a block of the modulatory effect of AP301 on ENaC. Though, differences were found in the EC_{50} values of amiloride. In the presence of AP301 a more than a 2.5-fold higher concentration of amiloride (25 ± 2 µM, n=7) was required to block the sodium current through ENaC in comparison to the concentration of amiloride (10 ± 1 µM, n=7) needed in the absence of AP301 (Shabbir et al. 2013). These results may demonstrate that amiloride has difficult access to its binding site when AP301 is also binding to ENaC.

Figure 10 B confirms that the expression of alpha, beta and gamma hENaC in the heterologous expression system was successful and identifies the measured sodium current in the HEK cells as the amiloride-senstive sodium current through ENaC. Results similar to those found in A549 are shown. The current was increased significantly after addition of AP301 to the bath solution whereupon this modulatory effect could be blocked by amiloride. These experiments illustrate for the first time that AP301 increases the amiloride-sensitive sodium current not only in cancer cells, where ENaC is overexpressed, but also in cell models, transfected with ENaC. This insight allows us to investigate the expression of ENaCs in the future and also its regulation pathways in heterologous cell systems.

3.3.2 EC_{50} value of AP301 in homologous and heterologous expression systems

To determine the half maximum of activation of AP301 for amiloride-sensitive sodium current through ENaC, alpha-, beta- and gamma-subunits of hENac were expressed in heterologous cell models. Human ENaC DNA was transfected into CHO and HEK cells and the modulatory effect of AP301 was analyzed. The results were compared with EC_{50} values found in A549 cells.

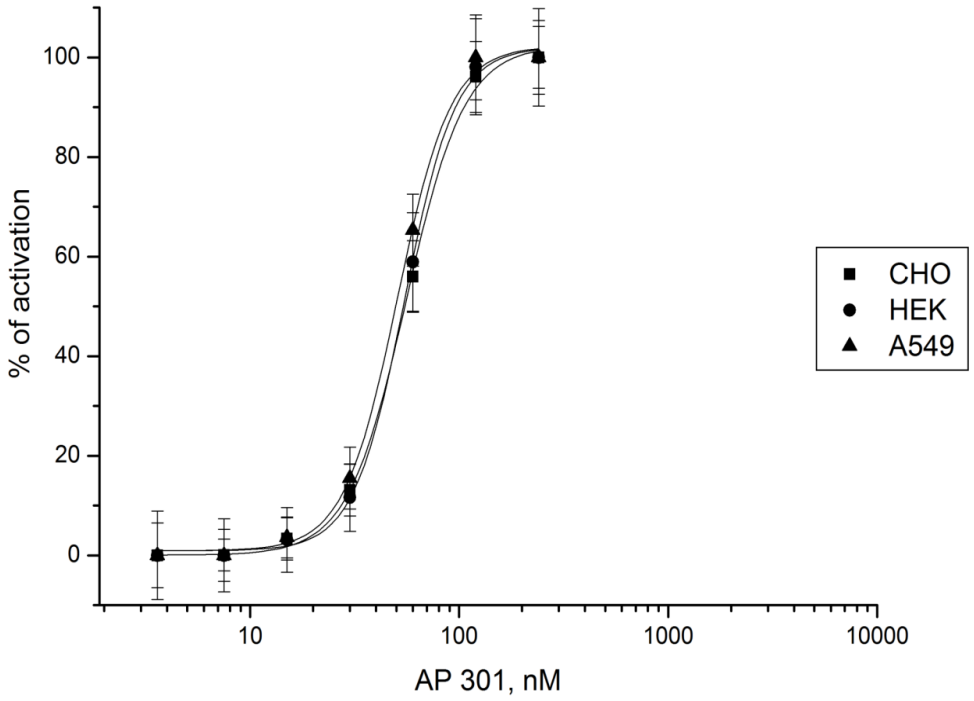

Figure 11: Comparison of EC50 values of AP301 in A549 cells and in heterologous expression systems: HEK and CHO

Dose-response experiments in whole-cell configuration, performed to determine the potency of AP301-induced current activation in transiently and endogenously expressed ENaC. Inward current was elicted at -100 mV. Mean values +/-SE for 7-11 experiments are given.

(Shabbir et al. 2013)

48 hours post transfection, whole-cell patch-clamp assays were performed. AP301 was dissolved freshly and was applied directly to the bath solution.

Figure 11 demonstrates that the EC_{50} value of AP301 is almost similar in A549, HEK and CHO cells (Shabbir et al. 2013). The apparent EC_{50} for AP301 activation of hENaC in nM was 55 ± 1 in A549 cells (n=11), 55 ± 2 when hENaC was expressed in HEK cells (n=7) and 58 ± 2 when hENaC was expressed in CHO cells (n=9) (Shabbir et al. 2013). These data clearly indicate that AP301 increases ENaC current in a similar efficacy and potency in all three expression systems (Shabbir et al. 2013).

3.4 Localizing the binding site of AP301 on ENaC

3.4.1 Interaction of AP301 with glycosylation sites at the extracellular domain of ENaC

The binding site of AP301 on the epithelial sodium channel is still unidentified (Hazemi 2011). However, in Hazemi's series of experiments, it was shown that it is expectable for AP301 to bind to glycosylations sites of the channel (Hazemi 2011). Since a cyclic disulfide heptadecapeptide (TIP17ox), derived from the lectin-like 17 amino acid domain of human TNF-alpha, was demonstrated to bind specifically to a dissacharide present in many glycan structures of glycoproteins, Hazemi wanted to investigate the theoretical interaction of the TIP peptides to sugar moieties on the cellular membrane in A549 cells (Hazemi 2011, Marquart et al. 2007). Therefore, she pretreated A549 cells with the enzyme PNGase F for achieving deglycosylation of the cell membrane (Hazemi 2011). Deglycosylation of A549´s cell membrane did not change channel conductivity but after addition of TIP peptides no activation of ENaC was to be seen, what indicates, that interaction of AP301 with ENaC requires precedent binding to glycosylated sites (Hazemi 2011).

3.4.2 Effect of AP301 on ENaC in inside-out and outside-out patches

Since it seems most likey that AP301 is binding to sugar moieties, which are found at the extracellular domain of ENaC, the next step was to perform outside-out and inside-out patch-clamp experiments in A549 cells. If the sodium current of ENaC would have been increased after adding AP301 to the bath solution during inside-out patch, it would have ment that AP301 binds against our expectations to the intracellular side of the channel.

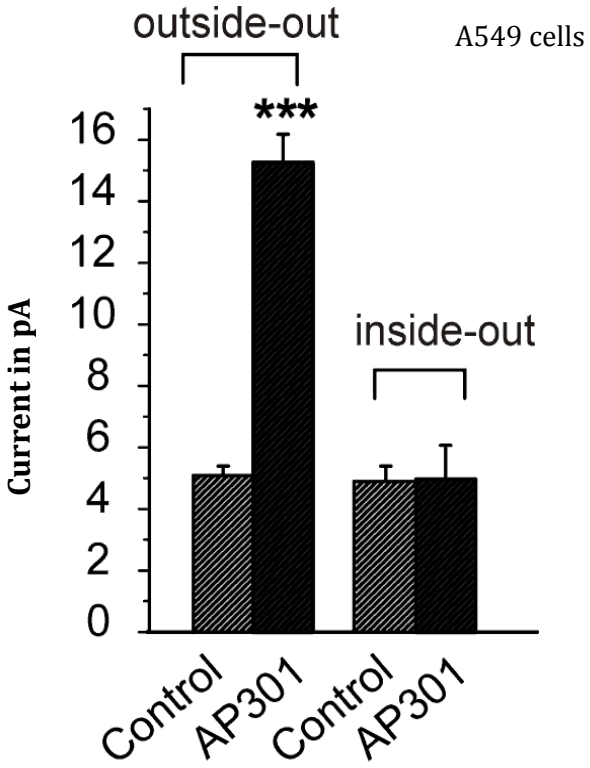

Figure 12: Localizing the binding site of AP301 at the extracellular domain of ENaC.
Outside-out and inside-out multichannel patch clamp experiments were performed in A549 cells. After a steady state of the control current, AP301 (120 nM) was added to the external solution. Inward current was elicted at -100mV (n=17).
(Shabbir et al.2013)

However, as shown in **Figure 12**, the control current (5 ± 1 pA) in outside-out patches was significantly increased (15 ± 1 pA) when adding 120 nM AP301 to the extracellular solution (Shabbir et al. 2013). In contrast, when performing inside-out patch-clamp experiments no increase of the current was to be seen after addition of AP301 to the external solution (Shabbir et al. 2013). These experiments confirmed that activation of hENAC by AP301 requires its interaction with the extracellular loop region of ENaC (Shabbir et al. 2013).

3.4.3 Subunit-specific modulation with AP301

With the help of the patch-clamp technique, we subsequently demonstrated that AP301 modulates the amiloride-sensitive sodium current in homologous and heterologous expression systems. Furthermore, there is great evidence that AP301 binds to sugar moieties, which are found on the extracellular loop of the epithelial sodium channel. Subsequently, this woke the interest, if AP301 modulates ENaC subunit-specific, since it is of great interest to localize the binding site of AP301 on ENaC. ENaC is composed of at least three homologous subunits – alpha, beta and gamma (Althaus et al. 2011, Canessa et al. 1994, Kashlan et al. 2011). Each subunit consists of two transmembrane domains (TM1 and TM2), a large extracellular loop and short intracellular amino and carboxyl termini whereupon the extracellular domain represents approximately 70% of the channel's mass (Bhalla et al. 2008, Kashlan et al. 2011). Some data suggest that individual ENaC subunits may have independent functions (Matthay et al. 2002). The sequence analogy of the three subunits amounts to 30-40%, whereupon it is supposed that the α-subunit is responsible for channel activity, whereas the β- and γ-subunits are required for channel expression at the membrane (Bhalla et al. 2008, Kashlan et al. 2011, Schild et al. 2010). The question arises, whether AP301 binds only to one of those three subunits or if it needs all subunits to be effective. Furthermore, it is of great value if AP301 enhances the probability of ENaC to remain open or if it enhances the expression of ENaC in the membrane, since the amount of sodium which attains into the cell through ENaC depends on two factors: on the number of ENaCs located at the membrane and on the open probability, which can be influenced by mechanical stress and various ligands (Eaton et al. 2010, Kashlan et al 2011). Hazemi demonstrated that is expectable that the rapid current increase affected by AP301 is caused by an increase in open probability, although an effect on channel expression cannot be excluded (Hazemi 2011).

To investigate the subunit-specific modulation of AP301, alpha-, beta-and gamma-subunits together, all subunits alone and dimeric combinations of hENaC's subunits were transfected into HEK cells.

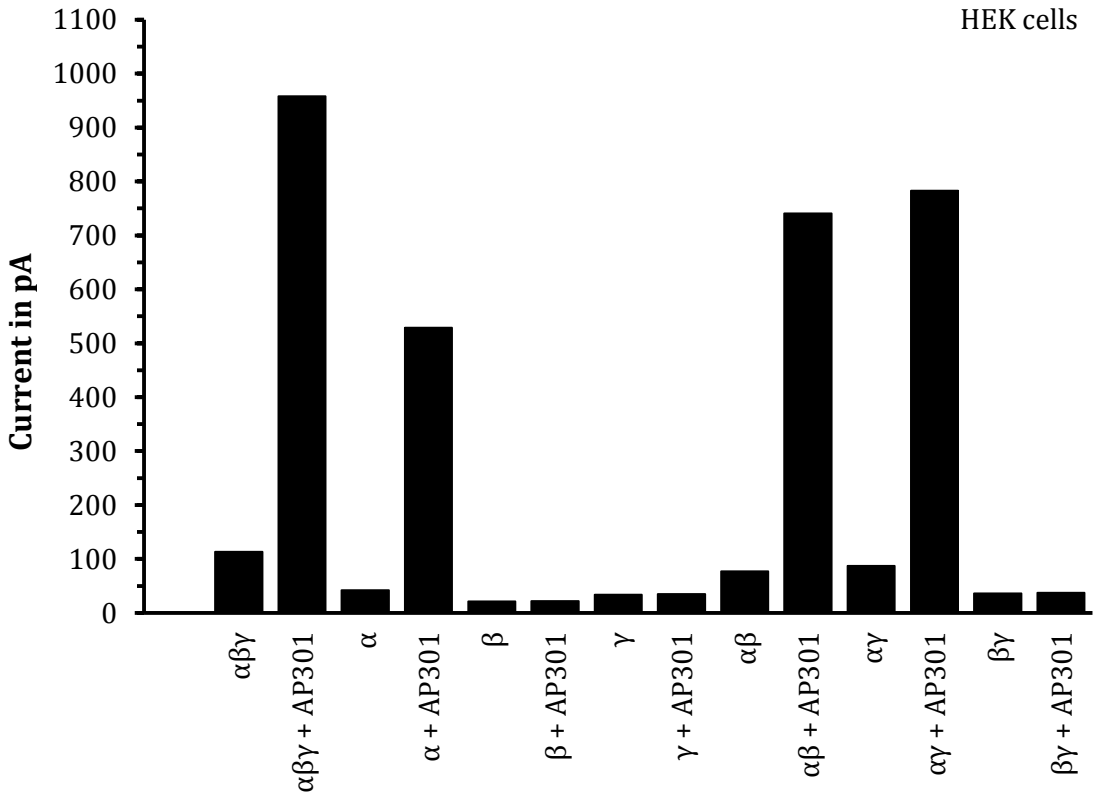

Figure 13: AP301 activation requires alpha-, beta- and gamma- subunit coexpression

Heterologous HEK cells were transfected with single and indicated combinations of hENaC subunits. The modulatory effect of AP301 (120 nM) was analyzed with whole-cell patch clamp assay. Inward current was elicted at -100 mV. Mean values +/-SE for 5-10 experiments are given.

Subunit	Control ENaC current (pA)	Current modulation with AP301 (pA)
αβγ	113 ± 3	958 ± 1
α	42 ± 7	529 ± 8
β	21 ± 9	22 ± 6
γ	34 ± 4	35 ± 9
αβ	77 ± 4	741 ± 3
αγ	87 ± 4	783 ± 5
βγ	36 ±11	37 ± 7

Table 2: Subunit-specific modulation of AP301 in HEK cells stable transfected with single and indicated combinations of hENaC subunits

Mean values of inward currents during control phase, following addition of AP301 to the bath solution (n=5-10).

Figure 13 and Table 2 show the results of the subunit-specific modulatory effect of AP301 on hENaC expressed in HEK cells. The first two bars show, as already demonstrated in **Figure 10**, that AP301 significantly increases the sodium current when all subunits are transfected together in HEK cells. Remarkably, an increase of the sodium current by AP301 is still achieved when only the alpha-subunit is expressed in the cells. Oppositional to this, the beta- as well as the gamma-subunit alone does not seem to be necessary for the modulation of AP301. Paradoxically, alpha- and beta- subunits transfected together as well as alpha- and gamma-subunits transfected together demonstrate a more enhanced current than observed when the alpha-subunit was transfected alone.

These results indicate, that AP301 has more than one binding site at the amiloride-sensitive epithelial sodium channel. The alpha-subunit seems to be the primary site of action for AP301 and at least one of its bindings sites must be found at the extracellular side of the alpha-subunit. But since the current is increased more when either beta- or gamma-subunits are transfected together with the alpha-subunit, it brings up the question to which extent beta- and gamma-subunits contribute to the enhancing effect and leads to the assumption that the different functions of all 3 subunits are needed for affecting modulation of AP301 on ENaC. We currently put forward the hypothesis that AP301 may induce these effects primarly at the α-subunit which is responsible for channel activity, whereas to a certain extent it also affects β-and γ-subunits which are required for channel expression at the membrane (Bhalla et al. 2008, Kashlan et al. 2011, Schild et al. 2010).

But to confirm this hypothesis, site-directed mutagenesis experiments should be the topic of future research. Since we expect AP301 to bind primarily with its lectin-like domain of TNF-alpha to one of the glycosylation sites on the extracellular side of ENaC's alpha-subunit the next step would include to mutate the glycosylation sites of the alpha-subunit, transfect one or a combination of these modified glycosylation sites into the HEK cell model and investigate the effect of AP301 with the help of patch-clamp technique. If de facto one of these glycosylation sites will disclose itself as the binding site of ENaC, no modulatory effect or at least a decreased modulatory effect of AP301 should be seen in patch-clamp experiments. Hence, this could also

reveal how many binding sites are substantially needed for affecting the maxium of the modulatory effect of AP301, but also to which extent each subunit with its binding sites contributes to this effect.

Moreover, it is necessary to confirm that AP301 increases the probability of ENaC to remain open and to investigate if activation of AP301 also somehow affects a greater insertion of the channel in the membrane. It was observed that the stimulatory effect of AP301 points rather to a direct effect on the channel kinetics in the plasma membrane than to an increase of the number of channels by inserting newly synthesized channels since that process would require tens of minutes (Carattino et al. 2003, Shabbir et. al 2013) However, not much is known about how ligand binding at the extracellular domain causes conformational changes in the pore (Kashlan et al. 2011). To answer this question it is first essential to understand the regulatory pathways of ENaC. Western blot experiments with ENaC's subunits transfected in HEK cells would give important new insights into the expression of diverse factors, which contribute to ENaC´s regulation pathways and hence may result in understanding the modulatory effect of AP301 on the amiloride-senstive epithelial sodium channel.

3.5 Conclusion

With the help of patch-clamp technique, it was shown that AP301 increases the sodium current through ENaC in a similar efficacy and potency in endogenous and in heterologous expression systems. Moreover, it was proved that AP301 increases exclusively the amiloride-sensitive sodium current through ENaC and that other ion channels do not contribute to this modulatory effect. Although the binding site of AP301 is still unknown, it was demonstrated on the one hand, that the specific interaction of AP301 with ENaC requires precedent binding to glycosylated extracellular loop(s) and on the other hand that even if the alpha-subunit seems to be the primary site of action, αβγ-hENaC subunit triplets are necessary to elicit a rapid and robust response to AP301.

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5 Appendix

5.1 Abstract

Pulmonary permeability oedema represents a frequent complication in a number of life-threatening lung conditions but apart from ventilation strategies, no specific therapy yet exists for its treatment. Since the epithelial sodium channel, ENaC, in the lung is the primary site for fluid reabsorption, increasing its activity presents promising drafts regarding the treatment of pulmonary permeability edema. TIP peptide AP301, which is presently undergoing clinical trials, was shown to enhance the sodium current of amiloride-sensitive epithelial sodium channels. But to date, the mechanism by which the TIP peptide AP301 activates ENaC-mediated sodium current remains elusive. Hence, the main aim of my diploma thesis was to investigate the modulatory effect of AP301 and its target specifity on ENaC.

A549 cells – which are a widely accepted model for exploring ENaC electrophysiology – were used for exploring the modulatory effect of AP301 on ENaC. With the help of patch-clamp technique, we confirmed that addition of AP301 to the bath solution results in a considerable increase of sodium flow through ENaC in A549 cells, which was blocked by amiloride. In order to demonstrate that the TIP peptide AP301, which mimics the lectin-like domain of TNF-alpha, directly affects the sodium flow through ENaC and not directly or indirectly other ion channels, ion substitution and inhibition experiments were conducted. It was shown, that replacement of sodium chloride in the bath solution with NMDG chloride resulted in a total abolition of the current enhancing effect of AP301, what exemplifies, that AP301 exclusively affects the sodium and not any other ion current. Furthermore, addition of CNG channel inhibitors, Zink and L-Cis-Diltiazem to the bath solution did not influence the current enhancing effect of AP301, what reinforced, that AP301 increases only the sodium current through epithelial sodium channels and that CNG channels do not contribute to this modulatory effect. Since it is known, that the activator of AMP-activated protein kinase, AICAR, inhibits epithelial sodium conductance in H441 monolayer cells, we investigated its effects on ENaC in A549 cells in order to show that AP301 has no effect, when ENaC is inhibited and hence confirm that AP301 does not affect other ion channels. After 1 hour pretreatment of A549 cells with AICAR, addition of

AP301 did not cause any enhancing effect, what proved that AP301 increases exclusively the amiloride-sensitive sodium current through ENaC.

Further, we sought to analyze the post-translational modification of ENaC and its possible effect on AP301 modulation in heterologous expression system. Therefore, we transfected ENaC DNA in HEK cells and repeated the experiments with AP301 and amiloride in order to investigate the modulatory effect of AP301 in cell models, which do not express ENaC endogenously. In order to facilitate laboratory work, we successfully achieved HEK and CHO cells, which stably expressed hENaC's subunits, by using G418 for selection pressure. To our knowledge, these experiments showed for the first time, that AP301 increases the amiloride-sensitive sodium current not only in cancer cells, where ENaC is overexpressed, but also in heterologous expression systems, what implicates the opportunity to investigate the expression of ENaC and also its regulation pathways in heterologous cell systems in the future. Moreover, dose-response experiments with amiloride in HEK cells, transfected with alpha, beta and gamma hENaC, were conducted, which demonstrated that in the presence of AP301 a more than a 2.5-fold higher concentration of amiloride was required to block the sodium current through ENaC compared to the amount of amiloride needed in the absence of AP301, what indicates that amiloride may have difficult access to its binding site, when AP301 is also binding to ENaC. To determine the half maximum of activation of AP301 for amiloride-sensitive sodium current through ENaC, dose-response experiments were performed in A549 cells and in the heterologous cell models CHO and HEK transfected with αβγ subunits of hENaC. The EC_{50} value of AP301 modulation was almost similar in A549, HEK and CHO cells, what indicates clearly that AP301 increases ENaC current in all three expression systems in a similar way.

The binding site of AP301 on the epithelial sodium channel is still unidentified, but it was shown previously, that it is most likely that AP301 is binding to sugar moieties, which are found at the extracellular domain of ENaC. Performing outside-out and inside-out patch-clamp experiments certified, that activation of hENAC by AP301 requires its interaction with the extracellular loop region of ENaC. Subsequently, this woke the interest whether AP301 binds only to one of the three subunits or if it needs

all subunits to be effective. To investigate the subunit-specific modulation of AP301, alpha-, beta- and gamma-subunits together, all subunits alone and dimeric combinations of hENaC's subunits were transfected into the HEK cell line. Patch-clamp experiments with AP301 revealed, that the alpha-subunit seems to be the primary site of action for AP301 and that at least one of its binding sites must be found somewhere at the extracellular side of the alpha-subunit. In contrast, it was observed that transfection of the beta- and of the gamma-subunit alone, as well as transfection of beta- and gamma-subunits together did not conduct any modulatory effect of AP301. Paradoxically, combinations of alpha- with beta- and alpha- with gamma-subunits resulted in a more enhanced increase in current than it was observed after modulation of the alpha-subunit alone. The fact that αβγ-hENaC subunits triplets showed the highest increase in current after modulation with AP301 demonstrates that all three subunits are necessary to elicit a rapid and robust response to AP301. Together these findings indicate that the specific interaction of AP301 with both endogenously and heterologously expressed ENaC requires precedent binding to more than one glycosylation site at the extracellular loop(s). However, to which extent each of ENaC’s subunit is involved in the modulatory effect of AP301 and which signaling pathways are influencing the activity of ENaC remains a topic for future research.

5.2 Zusammenfassung

Nicht-kardiale Lungenödeme resultieren aus einem Ungleichgewicht der Flüssigkeitshomeostase im epithelialen alveolaren Gewebe und stellen eine lebensbedrohliche Situation dar, für die bis heute noch keine geeignete Therapie zur Verfügung steht. Da der epitheliale Natriumkanal (ENaC) eine entscheidende Rolle in der Regulierung des Flüssigkeitshaushaltes der Lunge spielt, stellt seine Modulierung sowie die Erhöhung seiner Aktivität eine vielversprechende Therapiemöglichkeit für die zukünftige Behandlung von nicht-kardialen Lungenödemen dar. AP301 befindet sich derzeit in der klinischen Untersuchung zur Behandlung von Permeabilitäts-Lungenödemen und soll durch eine Erhöhung des Natriumstroms durch den ENaC in der Lunge zu einer Verbesserung der Flüssigkeitsklärung führen. Da der Wirkungsmechanismus von AP301 bis dato jedoch ungeklärt ist und die Suche nach seiner Bindungsstelle an besagtem Kanal Thema der aktuellen Forschung darstellt, beschäftigte ich mich im Zuge meiner Diplomarbeit mit der Erforschung der Modulation von AP301 am epithelialen Natriumkanal sowie mit seiner Targetspezifität, mit dem Ziel die Suche nach seinem Angriffspunkt am ENaC einzugrenzen. Die Experimente wurden an der Lungenkrebszelllinie A549, welche ENaC endogen überexprimiert, sowie an den Zellmodellen HEK und CHO, in welche ENaC erfolgreich stabil und dauerhauft transfisziert wurde, durchgeführt. Mit Hilfe der Patch-Clamp-Technik konnte gezeigt werden, dass AP301 den Amilorid-sensitiven Natriumstrom durch ENaC in A549 Zellen sowie in CHO und HEK Zellen gleichermaßen signifikant erhöht. Weiters wurde veranschaulicht, dass AP301 ausschließlich ENaC moduliert und keine weiteren Ionenkanäle zu diesem Wirkungsmechanismus beitragen. Zusätzlich konnten die Angriffspunkte von AP301 am epithelialen Natriumkanal eingegrenzt werden, indem einerseits gezeigt wurde, dass die Interaktion von AP301 mit ENaC eine Bindung an Glykosylierungsstellen, welche an der extrazellulären Domäne des Kanals zu finden sind, voraussetzt und andererseits, dass obwohl sich die Bindungsstellen von AP301 hauptsächlich an der alpha-Untereinheit zu befinden scheinen, für die schnelle, robuste und effiziente Erhöhung des Natriumstroms durch AP301 das αβγ Triplett notwendig ist. In welchem Ausmaß jede der drei Untereinheiten an dem Effekt von AP301 beteiligt ist

und welche regulatorischen Signalwege die Aktivität des epithelialen Natriumkanals beeinflussen, bleibt jedoch Thema der zukünftigen Forschung. Um den exakten Wirkmechanismus von AP301 am epithelialen Natrium Kanal nachvollziehen zu können, gilt es die Regulation des Kanals in den Fokus zu stellen, um so den Wirkmechanismus sowie den Angriffspunkt von AP301, welches einen Meilenstein in der Behandlung des nicht-kardialen Lungenödems darstellen könnte, näher zu kommen.

5.3 Curriculum Vitae

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Education

Oct 2007– ongoing	**Study of Pharmacy** at the University of Vienna Diploma thesis at the Department of Pharmacology and Toxicology *„Modulation of AP301 and its target speciftiy on the epithelial sodium channel (ENaC) in different cell systems“* (ao.Univ.Prof.Mag.Dr.Prof.Lemmens-Gruber)
Jun 2007	**A-levels („Matura“) with distinction** at BG|BRGPurkersdorf
Sep 1999 – Jun 2007	**Secondary school** at BG|BRGPurkersdorf

Relevant work experience

Aug 2013 – ongoing	**Regulatory affairs specialist** at Activartis Biotech Gmbh
Mar 2013 – Jul 2013 and Oct 2012 – Jan 2013	**Tutor of seminar “Pharmakologie, Pharmakotherapie und Toxikologie 2”**at the University of Vienna
Jan 2013	**Publication (***Pulmonary Pharmacology and Therapeutics.* **26**, S. 356-363**)** *“AP301, a synthetic peptide mimicking the lectin-like domain of TNF, enhancesamiloride-sensitive Na^+ current in primary dog, pig and ratalveolar type II cells”* Tzotzos S, Fischer B, Fischer H, Pietschmann H, Lucas R, Dupré G, Lemmens-Gruber R, Hazemi P, Prymaka V, Shabbir W

Language and IT Skills

Language skills	German – mother tongue English – fluent French – conversational ability Polish – conversational ability Latin – basic knowledge
IT	MS Office Programmes (Word, Excel, Powerpoint)