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in TNF- α induced activation“

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SUMMARY

Non-cardiogenic pulmonary edema is a complication within the lung due to a dysbalance of alveolar lung liquid clearance (ALC) and can be a life-threatening disease. The epithelial sodium channel (ENaC) at the apical surface of the alveolar epithelial cells plays a crucial role in maintaining ALC by working in conjunction with the basolateral located Na^+/K^+ -ATPase. A dysfunction of its activity reveals in impaired ALC and further leads to acute lung injury (ALI) and acute respiratory distress syndrome (ARDS).

To date, besides mechanical ventilation strategies no standard treatment exists. A promising strategy for therapy might be enhancement of ENaC activity and in this way increase ALC. It has been demonstrated that the lectin-like domain of the cytokine tumor necrosis factor- α (TNF- α) is able to activate the sodium uptake by amiloride-sensitive sodium channel in alveolar type II cells and thereby increases ALC.

However, the cellular mechanism of interaction is not cleared up. Studies have shown that the effect of TNF- α requires its lectin-like domain binding to the sugar moieties within the extracellular loop of α ENaC subunit. Hence, several cyclic peptides mimicking the lectin-like domain of TNF- α (TIP) were synthesized such as AP301, a TIP-peptide which has already passed clinical research phase II.

Therefore, the effect of TNF- α was studied in transiently transfected HEK293 cells by conducting whole cell patch clamp technique. And the role of the N-linked glycosylation sites was studied by site-directed mutagenesis. Moreover, the EC_{50} was examined.

The study indicates that the EC_{50} of TNF- α is slightly shifted to a higher concentration in α human ENaC mutants lacking one certain glycosylation site (N511Q) in comparison to human ENaC WT.

In conclusion, this research demonstrates a relation between TNF- α induced activation of ENaC and the N-linked glycosylation sites, but there is need for further testings after full deglycosylation.

ZUSAMMENFASSUNG

Das nicht-kardiale Lungenödem beruht auf einer Dysbalance der alveolären Flüssigkeitsclearance in der Lunge und kann eine lebensbedrohliche Erkrankung darstellen.

Der epitheliale Natriumkanal auf der apikalen Seite der alveolären Epithelzellen spielt in Zusammenarbeit mit der basolateralen Na^+/K^+ -ATPase eine entscheidende Rolle in der Aufrechterhaltung und Regulation des Flüssigkeitshaushaltes und ist verantwortlich für den vektoriellen Salz- und Wassertransport durch das alveoläre Epithel. Eine Dysfunktion dieses Natriumkanals äußert sich in Komplikationen, wie akute Lungenschädigung und Atemnotsyndrom.

Abgesehen von künstlicher Beatmung gibt es zur Zeit keine wirkungsvolle Therapie. Eine vielversprechende Lösung stellt die Wiederherstellung der alveolären Flüssigkeitsclearance durch Erhöhung der Aktivität des Natrium-Kanals dar. Es konnte gezeigt werden, dass das Zytokin, der Tumor-Nekrose-Faktor alpha mit Hilfe seiner lektin-ähnlichen Domäne die Natrium- Aufnahme über den amilorid-sensitiven Kanal in alveolären Typ II Zellen stimulieren kann und somit die alveoläre Flüssigkeitsclearance erhöhen kann.

Jedoch ist der Wirkmechanismus noch nicht vollständig aufgeklärt. Es gibt dennoch Hinweise, dass $\text{TNF-}\alpha$ mit seiner lektin-ähnlichen Domäne an Zuckerketten in der Extrazellulären Schleife der α -ENaC Untereinheit bindet. Daher wurden zahlreiche zyklische Peptide synthetisiert, die sich von der lektin-ähnlichen Domäne von $\text{TNF-}\alpha$ ableiten. Eines davon ist das Peptid AP301, welches inzwischen in der klinischen Prüfung die Phase II bereits erfolgreich durchlaufen hat.

Im Rahmen dieser Diplomarbeit wurde daher der Effekt von $\text{TNF-}\alpha$ in transient transfektierten HEK293 Zellen mittels whole cell Patch Clamp Technik untersucht und die EC_{50} ermittelt. Die Rolle der Zuckerketten bei der Aktivierung von ENaC durch $\text{TNF-}\alpha$ wurde mit Hilfe von Punktmutationen ermittelt. Die Ergebnisse zeigen, dass die EC_{50} der mutierten α Untereinheit (N511Q) im Vergleich zum Wildtyp (WT) leicht erhöht ist.

Zusammenfassend lässt sich daher sagen, dass ein Zusammenhang zwischen dem $\text{TNF-}\alpha$ induzierten Effekt in ENaC und den Zuckerketten besteht. Für eine definitive Aussage wären jedoch weitere Versuche mit einer kompletten Deglykosylierung notwendig.

1. INTRODUCTION

1.1. Pulmonary lung edema

Pulmonary edema is a consequence of a dysbalance of alveolar lung liquid clearance and one of the more common cause of admission to the hospital. It can be a life-threatening disease with a mortality of 30-50 % (Yang et al. 2010, O' Brodovich 2001, Matthay et al. 2002, Guidot et al. 2006).

This disease can be defined as increase in fluid in the lungs because of reduced active reabsorption from the alveolar space due to increased permeability of the membrane, epithelial lung injury, inflammation or downregulated ion transporter genes (Johnson et al. 2010). In the beginning, the excess fluid accumulates in the interstitial space of the lung which at first proceeds with no clinical symptoms. As soon as the fluid exceeds the ability of the interstitium to accomodate to the excess fluid, the fluid will flood the airspaces. This flooding leads to impaired gas exchange and in addition to acute respiratory distress syndrome (ARDS) (O'Brodovich, 2001). Typical symptoms which occur in patients with pulmonary edema involve shortness of breath, lung-crackling, pink-stained sputum, cough, anxiety, difficulty in breathing, restlessness and wheezing (Yang et al. 2010).

1.1.1. Classification

One can distinguish cardiogenic and non-cardiogenic pulmonary edema (Yang et al. 2010).

Cardiogenic pulmonary edema is a consequence of increased capillary hydrostatic pressure and also pulmonary venous pressure (Yang et al. 2010). This alteration is mostly induced by heart failure. The therapy of cardiogenic pulmonary edema consists of a symptomatic treatment of the impaired gas exchange with non-invasive ventilation strategies in order to improve alveolar liquid clearance combined with medical interventions (Yang et al. 2010, Berthiaume and Matthay 2007).

On the other hand non-cardiogenic pulmonary edema is defined as changes in permeability of the pulmonary capillary or alveolar epithelial membranes because of indirect or direct pathological processes like acute lung inflammation including sepsis, trauma, and severe pneumonia, which leads to acute lung injury (ALI) and the pathological extreme ARDS (Yang et al. 2010, Guidot et al. 2006). Besides ventilation procedures no standard therapy exists for non-

cardiogenic pulmonary lung edema (Yang et al. 2010). Therefore, it is necessary for novel developments to improve the clinical outcomes (Johnson et al. 2010).

All in all, it is indicated that the ability of the lung to clear edema liquid is essential to prevent outcome in both types of pulmonary edema and the life expectancy is reduced when the fluid reabsorption is impaired (Yang et al. 2010).

1.1.2. Alveolar lung liquid clearance

The key factor for recovery from ARDS is the ability to clear edema fluid and reconstitute a normal alveolar structure (Berthiaume et al. 1998).

Under physiological conditions a balanced alveolar liquid clearance (ALC) facilitates an efficient gas exchange in the lung across the air/blood barrier. It promotes exchange of oxygen from the airspace into the blood and CO₂ out of the blood (Matthay and Ware et al. 2001, Eaton et al. 2009). The efficient gas exchange depends on a thin liquid layer on the air facing side of the alveolar epithelium. Its amount has to be regulated precisely, otherwise gas exchange and alveolar function are affected (Eaton et al. 2009). The regulation is insured by a balanced passive secretion from the vascular space and a rate at which fluid is actively reabsorbed (Eaton et al. 2009).

Characteristic for ALI is a reduction of ALC, further cellular characteristics such as loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration and release of cytotoxic mediators (Johnson et al. 2010).

Especially the transepithelial neutrophil migration plays an important role by contributing to the membrane destruction and increased permeability of the alveolar capillary membrane. Their activation is stimulated by TNF- α , IL-1 β , IL-6, IL-8, IL-10, which are secreted by alveolar macrophages (Figure 1). The neutrophils are the primary cause for inflammation process by producing and releasing damaging pro-inflammatory and pro-apoptotic mediators. But ALI can also occur neutrophil-independent (Johnson et al. 2010).

It has been shown that the removal of alveolar fluid under normal conditions requires active ion transport mechanisms and an intact, functional alveolar epithelium (Matthay et al. 2002, Vergheze et al. 1999). Furthermore, the vectorial transport of salt and water across alveolar and distal airway epithelium through the amiloride-sensitive epithelial sodium channel (ENaC) on the apical surface is crucial for the fluid clearance and sodium is the dominant ion transport

mechanism involved in ALC (Berthiaume et al. 2007, Matthay et al. 2002) and is important in the pathophysiology of ALI (Berthiaume and Matthay et al. 2007).

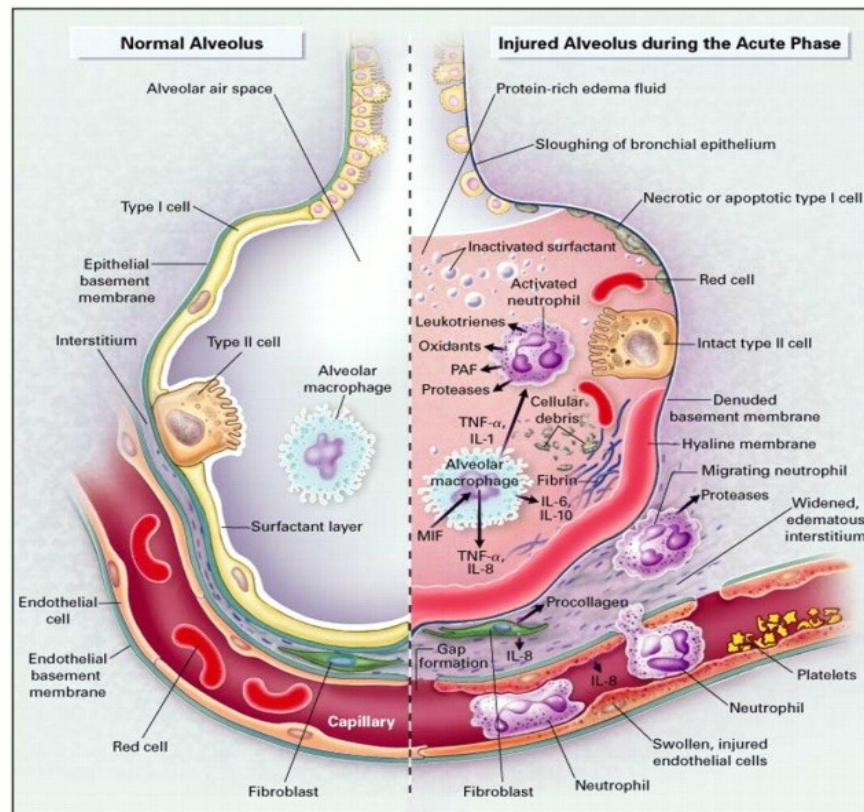


Figure 1: Normal alveolus on the left hand and injured alveolus on the right hand. (Bakowitz et al. 2012).

1.1.3. Different types of lung cells and their function

The pulmonary alveoli are composed of a thin alveolar epithelium (0,1-0,2 μm) that covers 99 % of the airspace surface area in the lung and contain two different types of cells (alveolar type I and alveolar type II cells) (Eaton et al. 2010). Both types of cells play a role in maintenance of the ALC (Guidot et al. 2006).

Type I cells which cover 95 % of the alveolar surface are large, thin, squamous cells . These cells prevent the movement of liquid and proteins from the interstitial and vascular spaces by forming a tight barrier to maintain relatively dry alveoli (Matthay et al. 2002). They exhibit cation and anion channels including ENaC. They contain highly selective and non-selective channels (HSC,

NSC), functional pimozide-sensitive cyclic nucleotide-gated channels (CNG), voltage-gated potassium channels, the cystic fibrosis transmembrane conductance regulator (CFTR) and the water channel aquaporin 5. The latter have the highest known osmotic water permeability of any mammalian cell type (Johnson et al. 2006), thus this indicates that there is some water movement across alveolar epithelial type I cells (Berthiaume et al. 1998).

The well studied type II cuboidal cells only cover < 5% of the internal surface of the lung. Their function is secreting pulmonary surfactant, epithelial repair and furthermore they are responsible for the vectorial sodium transport from the apical to the basolateral surface, which seems to be the driving force for fluid removal from the alveolar space (Matthay et al. 2002, Johnson et al. 2006, Berthiaume et al. 1998). They contain ENaC, Na^+/K^+ -ATPase, and CFTR which is primarily found in AII cells and conserves electroneutrality (Guidot et al. 2006, Johnson et al. 2006).

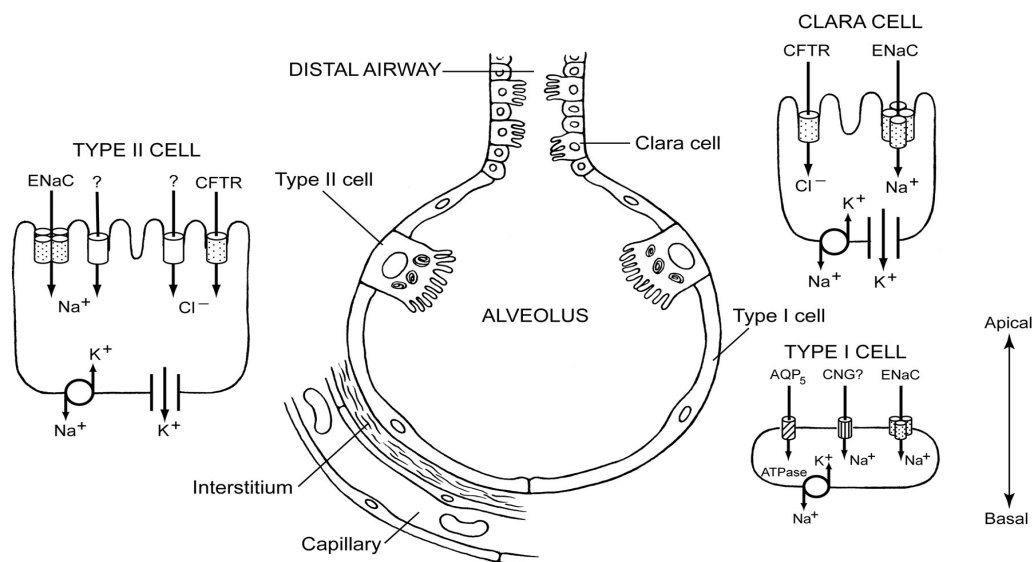


Figure 2: Schematic diagram of the distal pulmonary epithelium with different alveolar type cells (Matthay et al. 2000)

Besides the alveolar epithelium it has been shown that the clara cells in the distal airways absorb and transport sodium through the amiloride-sensitive sodium channel from the apical to the basolateral site (Matthay et al. 2002).

ENaC is expressed along the epithelium of the respiratory system in both types of alveolar epithelial cells (Berthiaume et al. 2007). This indicates that the ion and water transport proceeds across the entire alveolar epithelium (TI and TII-cells) (Johnson et al. 2006).

1.2. Epithelial Sodium Channel (ENaC)

Epithelial sodium channel (ENaC) is a member of the (subfamily) ENaC/degenerin gene family, a new class of ion channels, which first was discovered in 1990s (Kellenberger and Schild, 2002). ENaC and degenerin share sequence homology and have common functional characteristics (Kellenberger and Schild, 2002). The common functional attributes encompasses that they are Na^+ selective and amiloride-sensitive. Besides Na^+ , ENaC also conducts Li^+ -ions (Kashlan et al. 2011). Moreover, each associate is regulated by external ligands and/ or is mechanosensitive. ENaC is highly selective for sodium over potassium ($100 \text{ Na}^+ > 1\text{K}^+$) and is inhibited in sub-micromolar concentrations by the diuretic amiloride (EC_{50} of 150nM). Therefore, its synonyme is also amiloride-sensitive sodium channel. Furthermore, ENaC responds to external mechanical and chemical stimuli (Kashlan et al. 2011, Eaton et al. 2010).

1.2.1. Structure

ENaC is composed of three homologous subunits α, β, γ -ENaC. Whereby each subunit almost shares 30-40 % sequence homology with the others and has two hydrophobic membrane-spanning regions (M1 and M2) with short intracellular amino- and carboxytermini, and a large N-glycosylated extracellular loop (ECL) (Bhalla et al. 2008, Snyder et al. 1994). The three subunits are able to build a functional channel (Berthiaume et al. 2007).

Besides α, β, γ -subunits also a further subunit, δ -ENaC, has been described, which is able to build a functional cation channel by itself or in combination with (α), β, γ - subunits, because of its close homology to α -ENaC (Eaton et al. 2010, Kellenberger et al. 2010, Waldmann et al. 1995). It has already been successfully cloned in humans and monkeys (Ji et al. 2012). δ -ENaC is widely spread and can be found in epithelial tissues (lung, trachea, kidney, pancreas, liver, etc.), and besides also in nonepithelial tissues such as brain, heart, ganglion, placenta, and blood (Ji et al. 2012). Despite that fact, δ -ENaC seems to be mainly found in nonepithelial tissues (Waldmann et al. 1995). However, it has been shown that brain, testis, ovary and pancreas have the highest expression levels of δ -ENaC mRNA (Waldmann et al. 1995). Recently, *in vitro* studies in human airway epithelia cell lines and human alveolar epithelial type I-like cells have demonstrated that the protein expression in these cells is low and therefore it is assumed that δ -subunit does not contribute to the transepithelial sodium transport (Schwagerus et al. 2015).

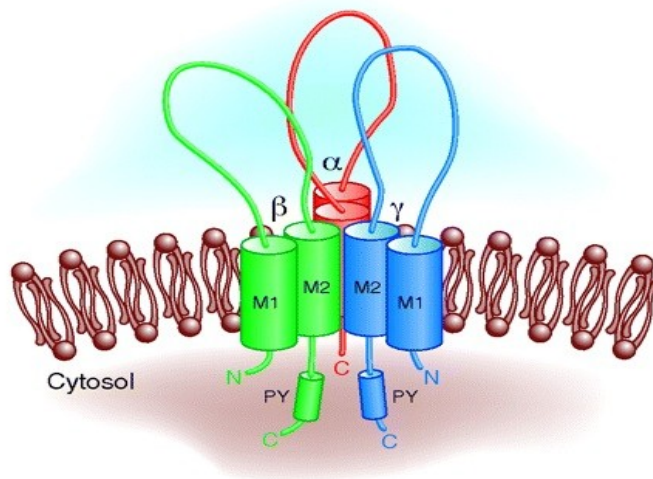


Figure 3: Structure of Epithelial Sodium Channel (ENaC) (Bhalla et al. 2008)

ENaC is assembled of three different subunits $\alpha\beta\gamma$. Each subunit contains two membrane- spanning domains (M1 and M2) with short intracellular amino- and carboxytermini and a large extracellular loop with the posttranslational modifications, such as N-glycosylation.

1.2.2. Location

In principle, ENaC is located at the apical membrane in many polarized epithelia cells in different tissues (Kellenberger et al. 2002, Bhalla et al. 2008). These cells embrace the epithelia cells in the urinary bladder, lung, airway, distal colon and ducts of salivary and sweat glands and also cells of the distal nephron in the kidneys (Bhalla et al. 2008).

1.2.3. Function

In mammals, the major role of ENaC is to mediate the sodium transport in epithelia and maintenance of the sodium homeostasis (Kellenberger and Schild. 2002). They are responsible for the vectorial transepithelial transport of Na^+ (Kellenberger and Schild, 2002).

The generated electrochemical gradient for Na^+ across the apical membrane, which is produced by the Na^+/K^+ -ATPase enables Na^+ to enter the cell via ENaC. Subsequently, the active transport across the basolateral membrane is carried out by the Na^+/K^+ -ATPase (Kellenberger and Schild,

2002) as shown in Figure 4. The vectorial transport of sodium is passively followed by water (Matthay et al. 2002).

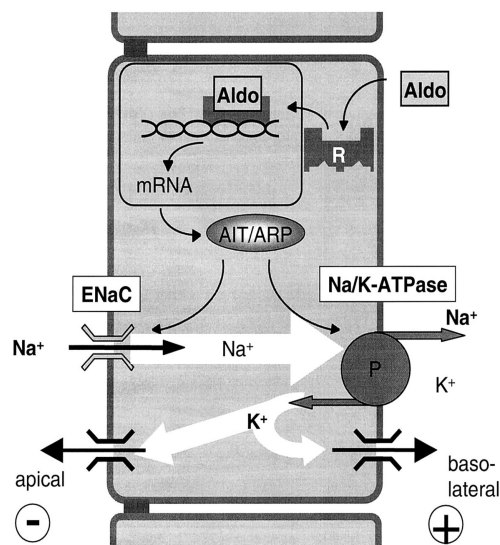


Figure 4: Sodium transport across the alveolar epithelial cell

ENaC located on the apical membrane works in conjunction with the Na^+/K^+ -ATPase on the basolateral membrane. The latter generates an electrochemical gradient which enables Na^+ to enter the cell via ENaC (Bhalla et al. 2008).

The described transport of Na^+ above is of particular importance for maintaining the composition and the volume of the fluid on both sides of the epithelium, the alveolar fluid in the lung or the saliva in the salivary glands (Kellenberger and Schild, 2002).

By contrast, the transepithelial sodium transport in the kidney is important for maintaining total body sodium balance and normal blood pressure (Eaton et al. 2010, Kellenberger et al. 2002).

To sum up, ENaC plays a major role in maintaining total body Na^+ homeostasis, blood volume, blood pressure, mucociliary clearance and lung fluid balance. Hence, any ENaC abnormalities may be correlated to several diseases in human body (Eaton et al. 2010, Kashlan et al. 2011).

For example the Pseudohypoaldosteronism Type I, which is a loss-of-function mutation of ENaC and results in excessive fluid accumulation in the lung and mild salt-wasting diuresis (Eaton et al. 2010). Further described ENaC mutations with decreased or increased ENaC activity, such as Liddle's syndrome (gain-of-function), cystic fibrosis and high-altitude pulmonary edema (Bhalla

et al. 2008).

1.2.4. Regulation of expression and activity

ENaC is regulated through extra- and intracellular factors respectively the number of channel expression at the cell surface as well as the channel open Probability (Po) (Sheng et al. 2006, Kashlan et al. 2011).

The number of channels in the apical membrane which transport sodium into the cell is determined by the balance between their rate of insertion and their rate of degradation (Eaton et al. 2009).

At it, the ubiquitination of ENaC is an important mechanism for the endocytosis of ENaC from the cell surface and further degradation (Bhalla et al. 2008). An enzyme cascade which encompasses following three enzymes, tags ENaC in ubiquitination process for degradation: ubiquitin-activating enzyme (E1), ubiquitin-conjugating-enzyme (E2) and the ubiquitin-ligase Nedd4-2 (neural precursor cell expressed developmentally down-regulated 4 protein) (E3). Nedd4-2 is responsible for the ENaC ubiquitination and mediates the final step. The protein-protein interaction between ENaC and Nedd4-2 occurs by Nedd4-2 binding with its Trp-Trp (WW) domain to the highly conserved proline-rich PPxY motif in ENaC, which is located at the C-terminus tail of each ENaC subunit (Eaton et al. 2009, Staub et al. 1996). Whereby the affinity can be altered through phosphorylation and so ENaC can be regulated directly and indirectly (Eaton et al. 2009, Bhalla et al. 2008).

First step of the enzyme cascade is the activation of the polypeptide Ubiquitin achieved by E1. Afterwards E2 binds to the activated Ubiquitin and lastly, Nedd4-2 (E3) ubiquitinates ENaC in the membrane (Eaton et al. 2009). Ubiquitin-molecules can be linked with each other to at least 4-5 ubiquitins resulting in a ubiquitin chain (Polyubiquitination) or are added to different lysin residues on the target protein (Monoubiquitination) (Eaton et al. 2009).

As soon as ENaC is ubiquitinated it will be internalized where the degradative pathway follows and depending whether the protein is poly- or monoubiquitinated it will either be degraded by proteasomes or ENaC which is modified with monoubiquitin will be targeted by the lysosomal degradative pathway (Eaton et al. 2009).

Moreover, patch clamp experiments have shown that there is another mechanism of regulation of activity which is proteolytic cleavage through intra- and extracellular proteases. So the proteolysis of ENaC subunits plays a major role in increasing Po and in addition activating

ENaC (Kleyman et al. 2009). The activation occurs by cleaving the ENaC subunits in specific sites within the extracellular domain (Bhalla et al. 2008, Kleyman et al. 2009). It has been shown for furin, a member of the pro-protein convertase family of serin proteases, that it cleaves α -subunit in two sites and γ -subunit in a single furin site. Mutations of these sites result in reduced sodium currents and therefore low Po (Kleyman et al. 2009, Bhalla et al. 2008). Studies indicate that processing of the γ -subunit has a dominant role for cleavage and further activation of the channel. So “near silent” ENaC and low Po are activated through serin proteases including furin, prostasin and other cellular proteases (Sheng et al. 2006, Kleyman et al. 2009).

By part, besides proteolytic cleavage the Po is also controlled by the temperature dependent Na^+ self-inhibition, a mechanism by which non-cleaved ENaC is inhibited due to a high extracellular Na^+ -concentration (Sheng et al. 2006, Bhalla et al. 2008). Studies demonstrated that Na^+ self-inhibition is relieved by furin-cleavage (Sheng et al. 2006, Bhalla et al. 2008). Moreover, it can be reduced by treatment with external proteases such as trypsin or by altering Na^+ -concentration from high to low (Sheng et al. 2006).

1.3. Dual role of TNF- α

The pleiotropic cytokine Tumor Necrosis Factor- α (TNF- α) is one of 18 members of the superfamily Tumor Necrosis Factor. Among others it is produced by monocytes, macrophages and lymphocytes. And one of its major roles is the inflammatory response and promoting the production of cytokines such as IL-1, IL-6, granulocyte-monocyte-colony-stimulating factor, IL-8 (Yang et al. 2010, Dagenais et al. 2004).

In comparison to the other cytokines, which only exert their activity by activating their receptors, TNF- α is exceptional because of its two different binding sites as shown in Figure 5. Apart from its regulary receptor binding site, which responds to both receptors TNF-R1 and TNF-R2, it has a special domain called lectin-like domain located on the tip of the molecule, which in particular binds oligosaccharides such as the disaccharide *N,N'*-diacetylchitobiose that are contained in many glycan structures of glycoproteins (Yang et al. 2010, Braun et al. 2005, Lucas et al. 2013, Marquardt et al. 2007). This domain differs regarding its location as well as its functions in different physiopathological processes (Yang et al. 2010). It is this structural feature which is responsible for the paradoxical effect of TNF- α (Figure 5).

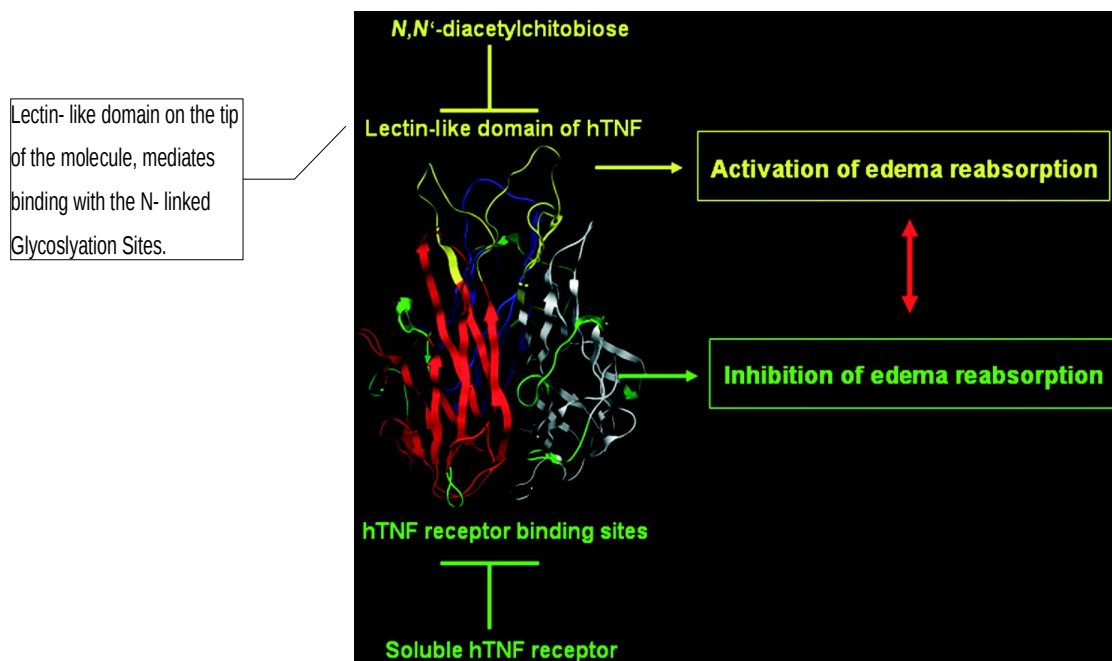


Figure 5: Dichotomous role of TNF- α (Braun et al.2005)

Showing two different binding sites of TNF- α . The yellow domain shows the lectin-like domain and the green part is responsible for the receptor binding.

Several studies have demonstrated the pro-inflammatory TNF- α induced effect during inflammatory process, which is mediated by its receptor binding site. One of these findings predicates that TNF- α decreases the expression of α -, β -, γ - ENaC subunits as well as the sodium uptake through amiloride-sensitive channel (Dagenais et al. 2004, Yang et al. 2010). Furthermore, TNF- α contributes to the pathogenesis of ALI by inducing acute pulmonary vascular endothelial injury and increasing pulmonary vascular permeability (Yang et al. 2010). Nevertheless, in contrast to that TNF- α also mediates a protective effect on pulmonary lung edema by enhancing the alveolar lung liquid clearance and activating edema reabsorption. Rezaiguia et al. (1997) have already studied this effect in rats with pneumonia. Moreover, studies in the alveolar epithelia cell line A549 cells have demonstrated that TNF- α enhances sodium uptake in a catecholamine-independent mechanism through amiloride-sensitive sodium channel by its lectin like domain (Yang et al. 2010, Braun et al. 2005, Fukuda et al. 2001).

For that cause several circular TIP Peptides, which mimic the lectin-like domain of TNF- α were developed (Figure 6b). These might be promising candidates for a potential treatment in patients with non-cardiogenic pulmonary edema, whereby the cellular pathway is not fully clear yet (Yang et al. 2010, Berthiaume 2003).

1.3.1. Structure of TNF- α

The protein TNF- α , which is secreted by macrophages is a 52 kDa homotrimer and consists of 157 amino acid residues (Baldwin et al. 1996). TNF- α has three chains called A, B and C (PDB). The sequence of amino acid residues from C101-E116 build the lectin-like domain on the tip of the molecule. In contrast to that, the receptor binding site is located on the basolateral region of the molecule (Hazemi et al. 2010).

Moreover, TNF- α has a single, intramolecular disulfide bridge between residue cysteine 69 and cysteine 101 (Eck et al. 1989).

The disulfide bridge is unstable and therefore it is not desirable for therapeutical use (Hazemi et al. 2010). So in the further designed TIP- Peptides this certain disulfide bridge is missing whereas the amino acid residues T105, E107 and E110 seem to be essential for the activating effect of ENaC through TNF- α (Hazemi et al. 2010). Their importance has been studied in A549 cells by generating mutations and replacing these residues with Alanin. The effect on sodium current was lost in comparison to the peptides where these residues were retained (Hazemi et al. 2010).

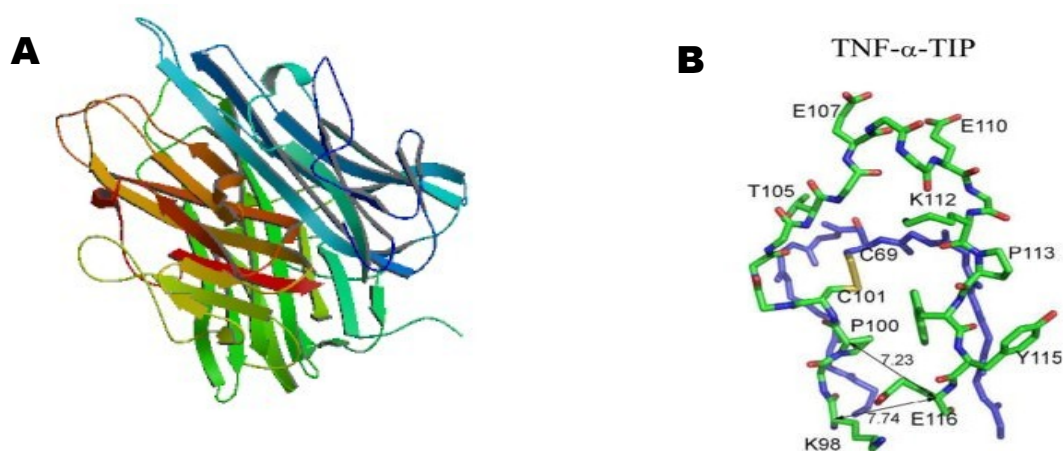


Figure 6:

(A) Structure of TNF- α at 2.6 Å resolution (PDB)

(B) 3D- structural model for human TNF- α as a template for further designed TIP- Peptides (1a8m template PDB) (Hazemi et al. 2013).

2. AIM OF STUDY

The role of TNF- α in alveolar lung liquid clearance and sodium transport across the alveolar epithelia has been investigated in several studies, for example in rats with acute bacterial pneumonia (Rezaiguia et al. 1997). Fukuda et al. (2001) demonstrated in their studies the mechanism by which TNF- α enhances alveolar lung liquid clearance in rats and increases the sodium transport in isolated human alveolar epithelial cells (A549) through amiloride-sensitive sodium channel.

Since the physiological role of ENaC in maintaining lung fluid balance in the lung has been demonstrated in mice, it is not too far to seek that studies in increasement of ENaC activity are in the focus of research (Hummler et al. 1996, Berthiaume et al. 1998). To date, no standard treatment for non-cardiogenic pulmonary edema exists, wherefore there is need for the development of novel therapies.

Studies indicate that ALC can be upregulated with the lectin-like domain of TNF- α . Consequently, several TIP-peptides mimicking the lectin-like domain of TNF- α by using it as a template were developed, such as AP301. This TIP-peptide has passed clinical research phase II and its ability to enhance ENaC activity has been demonstrated in alveolar epithelial type II cells of dogs, pigs and rats (Tzotzos et al. 2013).

However, the mechanism of interaction is still not clear. Our laboratory suggests that AP301 targets the N-linked glycosylation sites and requires those for activation of ENaC before binding to a specific binding site, whereby N511 plays a crucial role. After complete deglycosylation the effect of TNF- α and AP301 should get lost, which has been demonstrated in a heterologous (HEK293) and endogenous (A549) expression system (Shabbir et al. 2013, Hazemi 2011). Hazemi (2011) studied the same effect for mouse TNF- α (mTNF- α) in A549 cells.

Hence, the aspects of this study was to analyse the effect of TNF- α on α , β , γ -human ENaC (hENaC) WT and the mutation of the α N232Q and α N511Q -hENaC subunit with β -, γ -hENaC in a heterologous expression system (HEK293 cells) by determining the EC₅₀ in whole cell patch clamp experiments. Furthermore, the aim was to investigate the importance of the N-linked glycosylation sites with the aid of site-directed mutagenesis in order to support previous studies that N-glycosylation sites within the ECL are crucial for interaction with ENaC.

3. MATERIAL AND METHODS

3.1. Cell culture

All experiments were carried out in human embryonic kidney 293 cells (HEK293) (Passage 5-25), which were purchased from American Type Culture Collection (Manassas, VA).

This cell line is used as a heterologous expression system which does not express the amiloride-sensitive sodium channel endogeneously and therefore it is qualified for transfections with any desired ENaC subunit combinations.

The whole work of the cell culture was conducted under aseptic conditions. For this purpose only sterile medium, pipettes and cell culture flasks were used. For culturing the cells, Dulbecco's modified Eagle's medium (DMEM, Table 1) was used, supplemented with 10% fetal bovine serum (FBS, Table 1) and 1% penicillin-streptomycin (Pen/Strep, Table 1) which is essential for the proliferation. The cells were cultured in tissue flasks in the incubator at 37°C with a humidified atmosphere of 5% CO₂.

3.1.1. Splitting and seeding cells

The cells were daily observed under the microscope to check their confluence. Every two to three days they reached a confluence from about 70-90 % and the cells were splitted with a splitting rate 1:10.

At first the medium was aspirated from the edge of the culture flask. Afterwards, the cells were washed gently with PBS without Ca⁺/Mg⁺ (Table 1). To detach the adherent growing HEK293 cells from the surface of the culture dish they were incubated with 500µl/0.5ml Trypsin-EDTA (Table 1) at room temperature (RT), right after aspirating the washing solution PBS. Around 5 minutes later the enzyme digestion was stopped by adding the pre-warmed medium into the flask and resuspending the cells. For a splitting rate 1:10, 500µl/0.5 ml of the cell suspension were added into sterile T25 culture flasks with 4.5 ml of fresh medium.

Additionally, 24 hours before patch clamp experiments were planned, 90- 140 µl (per two dishes) cell suspension depending on the confluence of the HEK293 cells were seeded in 35mm tissue culture dishes with 2 ml culture medium. At last, the dishes as well as the flask were maintained at 37°C in a humidified atmosphere of 5% CO₂.

Table 1: Cell culture reagents

Name	Provider
Dulbecco's modified Eagle's medium/ nutrient mixture F12 Ham DMEM-F12	Sigma Aldrich GmbH
Fetal bovine serum (FBS)	Sigma Aldrich GmbH
Penicillin-Streptomycin	Sigma Aldrich GmbH
Phosphate buffered saline (PBS)	Gibco (Life Technologies)
Trypsin- EDTA (0,05%)	Gibco (Life Technologies)

3.1.2. Freezing cells

For cryopreservation of cultured cells an appropriate freezing medium was required. The freezing medium had to be prepared freshly and could not be reused. It contained 50% DMEM (serum-free medium), 30 % FBS and 20% of the cryoprotective agent DMSO. Until use it was stored in the freezer (-20 °C).

The cryovials (from Sterilin) for the freezing were labelled (cell line, passage number, date) and stored on ice under the laminar. The growth medium was aspirated and discarded. After washing the attached cells with PBS and afterwards aspirating, the cells were detached with trypsin and incubated at 37°C . The cells then were resuspended with 3 ml DMEM and the whole content was filled into a tube and centrifugated for 5 min at 3000 RPM. After centrifugation one could see a pellet on the ground and the supernatant was aspirated. The cell pellet was resuspended with growth medium. 750µl cell suspension were allocated in each cryovial and resuspended with 750µl freezing medium. In order to cool down the cryovials, they were kept on ice for 5 more minutes and then placed in a freezing container containing a isopropanol chamber and then stored at -80°C. The freezing container allows decreasing the temperature approximately 1°C per minute.

After seven days one cryovial was thawed and cultivated to test whether the freezing was successful and if the cells did survive. Finally, the rest of frozen cells were transferred into the liquid nitrogen storage, which enables a long term storage of the cryovials.

3.1.3. Thawing cells

The cryovials were removed from the liquid nitrogen storage and transported on ice. Subsequently, they were rapidly thawed by dunking them into the waterbath without submerging the cap until only a small globuli of ice remains in the vial. The next steps were carried under the laminar. The cell suspension in the cryovial was transferred into a tube. 8 ml growth medium was added and afterwards the mixture was centrifugated for 5 minutes at 4000 RPM. The supernatant was aspirated and the cell pellet was left on the ground and then resuspended again with growth medium. In the end, the cell suspension was conveyed to a T25 tissue culture flask with fresh medium and finally maintained in the incubator at 37°C.

3.1.4. Transfection

In order to perform patch clamp experiments in HEK 293 cells, they were transiently transfected with cDNA encoding for α -, β -, γ - human (h) ENaC WT or site-directed α -hENaC mutants in co-expression with $\beta\gamma$ -h ENaC WT (Table 2).

The cells were seeded 24 hours before transfection was carried out. Firstly, the cells were observed under the microscope to control their growth and to check whether they were contaminated or not. Furthermore, for the patch clamp experiments the cells needed to be single colonies, so their distribution was observed. Meanwhile the transfection reagent X-treme GENE HP (-20°C, Table 2) was pre-warmed at RT, so as the serum-free medium (without FBS and Pen/Strep) (+4°C).

For each transfection 70 μ l serum free medium was filled into a round-bottom-glass tube. Per one dish, 500-700 ng DNA of each subunit was first vortexed and then mixed with the medium in the glass tube. To get a homogeneous solution the glass tube was closed and vortexed again. If Snyder's cDNA was used then a green fluorescent protein marker (GFP, Table 2) was added to the mixture in order to make sure that the transfection was successful. So the cells could later be surveyed under the fluorescence microscope. In the end 3-4 μ l of the transfection reagent was attached directly to the solution. Afterwards, the glass tube was rubbed between the hands and then incubated for 20 min at RT. The second part of the transfection consisted of usual cell culture steps. After labeling the petri dishes the medium was aspirated and the cells were washed slowly with PBS. The PBS was then removed totally and the prepared DNA-solution was fastly dripped drop by drop into the dish. 2 ml DMEM was added and the dish was incubated for 48

hours at 37°C in a humidified atmosphere of 5% CO₂.

Table 2: DNA and transfection reagent

Name	Provider
enhanced green fluorescent protein tagged cDNA encoding α -, β -, γ - human (h) ENaC	Dr. Deborah L. Baines (St. Georges's University of London, UK)
cDNA encoding α -, β -, γ -h ENaC	Dr. Peter Snyder (University of Iowa Carver College of Medicine Iowa City, IA)
cDNA encoding α N232Q, α N511Q	Department of Pharmacology and Toxicology, University of Vienna
X-treme GENE HP Transfection Reagent	Roche Diagnostic GmbH, Mannheim
Ref. Nr.: 06366236001)	Germany

3.2. Molecular biology

3.2.1. Transformation

Transformation is a procedure by which bacteria cells are used as a transient host to reproduce DNA. In this case DH5 α competent bacteria cells (purchased from Invitrogen, stored at -80°C) were used as transient hosts. After thawing the cells on ice they were gently mixed with the pipette tip and aliquots of 50 μ l were transferred into a 1.5 ml microcentrifuge tubes, which were placed before on ice. Each microcentrifuge stands for one transformation. Unused cells were refreezed again at -80°C. 1-5 μ l of the DNA which was supposed to be duplicated was added to the cells and mixed gently. Finally, the mixture in the tube was incubated on ice. After 30 min on ice the cells were heat shocked for 20 seconds in a 42°C water bath by solely dipping them into the water bath without shaking. Then, for another two minutes the cells were placed on ice again. Afterwards, 950 μ l of SOC-Medium (Table 3) was added to each tube and incubated at 37°C for 1 hour at 250rpm.

The cells were spread on the pre-warmed selective LB-agar-plate containing 100 μ g/ml either kanamycin or ampicillin (depending on the DNA) and incubated overnight at 37°C. 16 hours later enough single colonies should be visible on the plate. Single colonies were picked with a pipette tip and placed together with the pipette in a glass tube containing 3 ml LB-medium (Table

3) and the appropriate antibiotic (300µl in 100ml) for each transformation. Lastly, they were incubated in the bacterial incubator at 37°C with 250 rpm for 16-18 hours.

In order to have a back up and facilitate the work glycerol stocks were made. Therefore, 900µl of the culture together with 900 µl glycerol were transferred into an eppi and inverted for several times until a homogenous mixture was obtained. Afterwards the eppi was stored in the freezer at -80°C.

Table 3: Transformation reagents and composition

SOC medium (+4°C)	20g tryptone
	5 g bacto Yeast extract
	0.5 g NaCl (10 mM)
	0.2 g KCl (2.5 mM)
	added to ddH ₂ O to a final volume of 1L
	autoclaved with 1 bar
	completed with
	10 ml 1 M MgCl ₂
LB broth (+4°)	10 ml 1M MgSO ₄
	10 ml 2 M glucose
LB agar (+4°C)	20 g LB broth
	added ddH ₂ O to a final volume of 1 L
	autoclaved
LB medium (+4°C)	20 g LB broth
	15 g agar
	added to ddH ₂ O to a final volume of 1L
	autoclaved
LB medium (+4°C)	10 g bacto- tryptone
	5 g yeast extract.
	10 g NaCl
	added to H ₂ O to a final volume of 1L
	adjustment of pH to 7.5 with NaOH

All chemicals and reagents were purchased from Sigma-Aldrich GmbH.

3.2.2. DNA-Extraction

As soon as the glass tubes were showing a blur the whole content was transferred into eppis and centrifugated for 2 min/12000rpm. The DNA-Extraction was realized with the GeneJET Plasmid Miniprep Kit by Thermo Scientific (#K0503). The detailed protocol for the DNA-Purification can be found in the Producer's Product Information Sheet :

https://tools.lifetechnologies.com/content/sfs/manuals/MAN0012663_GeneJET_Genomic_DNA_Purification_UG.pdf

In the end, the concentration of the purified plasmid DNA was measured with the NanoDrop and was then finally stored in the freezer at -20°C.

3.3. Electrophysiology

3.3.1. Whole cell patch

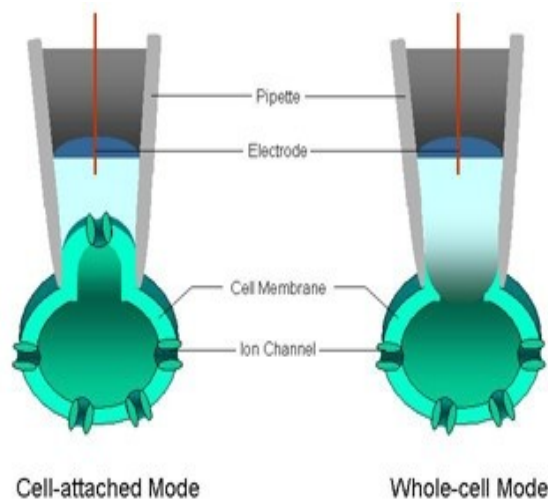
The whole cell configuration is one of the various ways to patch a cell. It allows access to the interior cell and enables measurements of the current throughout the whole cell. The other three patch configurations are cell attached, inside out, and outside out (Hamill et al. 1989).

To obtain a giga seal a single cell was chosen and the pipette was carefully approached to the cell until finally gently contacting the cell with the tip of the pipette without squashing it. Next a slight suction was applied with the syringe. As soon as the cell was attached the seal resistance increases and changes from MΩ to GΩ. By giving more suction or voltage pulse to the cell the membrane is ruptured and the interior of the cell comes into contact with the pipette solution as shown in Figure 7. The whole cell mode is created and in the following currents through all the ion channels in the whole cell can be recorded.

Figure 7 : Cell attached and whole cell mode in patch clamping

Cell attached mode allows to study single ion channels. Whole cell mode allows measurements of currents of all the ion channels in one cell.

(Bo Skaaning Jensen, NeuroSearch, Denmark)



3.3.2. Pipettes

Patch pipettes were made from thin-walled borosilicate pipettes (Harvard Apparatus, Holliston, MA). They were pulled and heat-polished with the DMZ Universal Puller (Zeitz Instruments, Martinsried, Germany) to obtain a resistance between 3 to 12 M Ω .

The pipettes were filled with the pipette solution by syringe and needle (Table 4). Snapping with the finger against the pipette helped to get rid of the air bubbles. Before using the pipettes the resistance was controlled. Pipette resistances lower than 2 M Ω were discarded as well as pipette resistance higher than 12 M Ω . For each cell a fresh pipette was used.

3.3.3. Whole cell recording in HEK293 cells

The effect of TNF- α on the amiloride-sensitive sodium channel was examined in transiently transfected HEK293 cells after 24-48 hours transfection at RT (19-22°C).

All the experiments were carried out with patch clamp by using an Axopatch 20B amplifier and DigiData 1440A with *pCLAMP10.2* software (Axon Instruments, Union City, CA). The HEK293 cells were patched in the whole cell configuration as described above at a holding potential (E_h) of -100 mV. Data acquisition and storage were processed directly to the PC.

In avoidance of patching non transfected cells, the cells were firstly observed under the fluorescence microscope whether they were green or not because of the green fluorescence protein tag in the cDNA. Afterwards, the growth medium was replaced with 2 ml bath solution (Table 4).

The cells were totally submerged. In order to blunt Na^+ self-inhibition and increase P_o , the cells were treated with 100 μl trypsin and incubated for 5 minutes at RT. Trypsin was then washed out and the dishes with the cultured cells were placed on the stage of an inverted microscope (Axiovert100; Carl Zeiss, Oberkochen, Germany).

When membrane was attached the resistance of the pipette increased and with subsequent suction a “gigaohm seal” was obtained, which indicates that the contact between membrane and pipette was stable. With a slight suction the membrane was ruptured. After the equilibration period of 5 min the cells were clamped at a holding potential of -100 mV and control currents were recorded. Whole cell currents were filtered at 5 kHz and sampled at 10 kHz. After control recordings aliquots of a TNF- α stock solution was cumulatively added into the bath solution starting with 2.5 nM (5 nM, 10 nM, 20 nM) and finally 40 nM until steady state was achieved. The wash-in phase lasted about 1 min until first effects could be observed. The same experimental protocol as during the control recordings was applied for each concentration of the test compound TNF- α .

In the end, when steady state was achieved amiloride hydrochloride (10 μl - 100 μl) was added to block ENaC in order to prove if the observed effects in increase were due to the amiloride-sensitive sodium channel.

Concentration-response curves and EC_{50} values were fitted and estimated for currents recorded at Eh of -100 mV by using Microcal Origin 7.0 (OriginLab, Northhampton, MA).

Table 4: Patch clamp solution and composition

Pipette solution (+4°C)	135 mM potassium methane sulphonate 10.0 mM KCl 6.0 mM NaCl 1.0 mM Mg ₂ ATP 2.0 mM Na ₃ ATP 10.0 mM HEPES 0.5 mM EGTA adjusted to pH 7.2 with 1 M KOH filtered with a pore size equivalent to 0,2 mm diameter
Bath solution (+4°C)	145 mM NaCl 2.7 mM KCL 1.8 mM CaCl ₂ 2.0 mM MgCl ₂ 5.5 mM glucose 10 HEPES adjusted to pH 7.4 with 1 M NaOH solution

All solution ingredients were purchased from Sigma-Aldrich GmbH.

3.4. Test compounds

The test compound TNF- α , which was used in the experiments was obtained from Sigma-Aldrich, Saint Louis, USA. A recombinant mouse TNF- α , which was expressed in *E. coli* was used. The effect of the test compound was studied in HEK293 cells in concentrations ranging from 2.5–40 nM. A stock solution was prepared with distilled water and stored in the freezer at -20°C.

Furthermore, amiloride hydrochloride hydrate (purchased from Sigma Aldrich GmbH) was used to block the sodium current through amiloride-sensitive sodium channel in concentrations ranging from 10-100 μ M.

3.5. Statistical analysis

Data in figures are given as the mean $\pm$ S. E. .The experiments were performed on three to nine patches of independently transfected cells in a heterologous expression system. Comparisons between whole cell currents before and after TNF- α treatment in the ENaC-WT and α -ENaC-mutation co-expressed with β -, γ -WT were determined with an unpaired, two-tailed t-test by using GraphPad Prism software (version 3.02; GraphPad Software, Sand Diego, CA).

A value of $p < 0.05$ was considered as statistically significant. Dose-response curves were plotted and EC₅₀ values were determined using Microcal Origin 7.0 (Origin Lab, Northhampton, MA).

4. RESULTS

The capability of TNF- α to enhance sodium current through amiloride-sensitive sodium channel has already been investigated in A549 cells (Hazemi et al. 2010). This cell line has many characteristics in common with alveolar type II cells and expresses α , β , γ - ENaC mRNA endogenously (Lazrak et al. 2000). While in the heterologous expression system (HEK293 cells) no mRNA encoding α -, β -, γ - subunits could be proven (Ruffieux-Daidié et al. 2008). Therefore, it is a widely accepted and convenient model for expression studies of any desired subunit combinations.

To determine the effect on ENaC induced by mTNF- α and the importance of the N-linked glycosylation sites, HEK293 cells were transiently transfected either with plasmids coding for α -, β -, γ -human ENaC WT or with α -subunit mutants expressed with β -, γ -hENaC WT. Although the α subunit is able to build a conducting pore by itself the expression can be increased by over 100fold if it is co-expressed together with β - and γ - subunits (Benos et al. 1999, Canessa et al. 1994a).

To determine the effect of glycosylation sites the α -subunit mutants lacked one certain N-glycosylation site (N232Q or N511Q). Therefore, our lab generated mutations in a site-directed mutagenesis where two different Asparagine within the ECL were substituted to Glutamine. As a result no glycosylations are build, whereby the lacking glycosylation sites should not affect the function of ENaC in any manner (Snyder et al. 1994).

Each $\alpha\beta\gamma$ hENaC subunit contains a short cytosolic chain, as well as a large ECL with numerous glycosylation sites. Canessa et al. (1994b) described the glycosylation sites in rat ENaC (rENaC) as shown in Figure 8. Furthermore, each subunit contains a different number of glycosylation sites, thus in α six glycosylation sites can be found and twelve and five in β and γ (Figure 7).

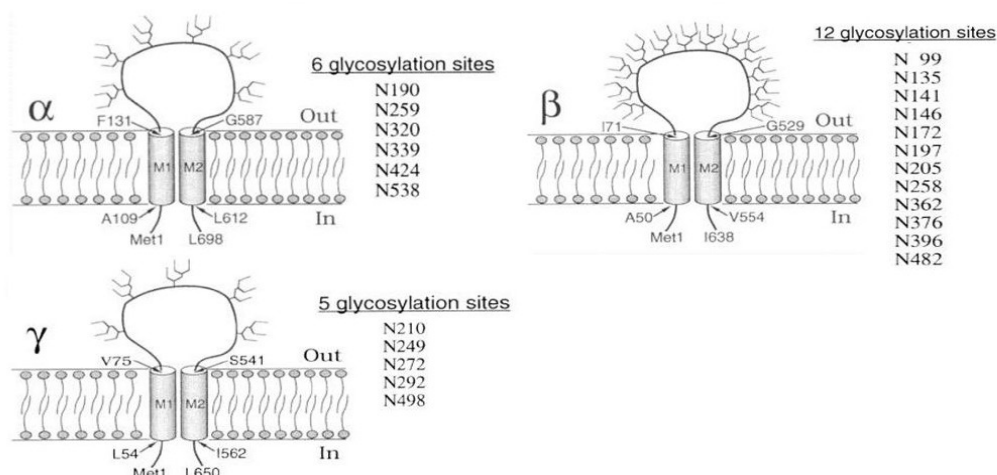


Figure 8: Membrane topology of rENaC (Canessa et al. 1994b)

The extracellular loop of each subunit contains a different number of glycosylation sites. There are 6 present for α , 12 for β and 5 for γ .

In the ECL of human α ENaC there are 5 glycosylation sites (N232, N293, N312, N 397, N511) located. Hession et al. (1987) demonstrated that glycoproteins bind to TNF- α , wherefore it is assumed that the interaction of TNF- α with ENaC requires those glycosylation sites. Studies in A549 cells have confirmed this assumption (Hazemi et al. 2011).

The effect on the amiloride-sensitive sodium current was measured with patch clamping technique in the whole cell mode. Previous studies of ENaC in A549 cell line have shown that the sodium inward current at a holding potential of -100 mV enhances at its maximum. Therefore, experiments were performed at E_h of -100 mV (Tzotzos et al. 2013). Firstly, the cells were incubated with 100 μ l trypsin for 5 minutes to activate non-cleaved ENaC and increase their P_o (Hughey et al. 2004). After trypsin was washed out the control channel activity was recorded. Subsequently, the test compound was cumulatively added in concentrations ranging from 2.5 nM to 40 nM into the bath solution. As soon as steady state effect was obtained the ENaC blocker amiloride was added to prove that the recorded sodium current was mediated by the amiloride-sensitive sodium channel.

4.1. Concentration-dependent activation of human α WT ENaC through TNF- α

Figures 9 and 10 show the effect of TNF- α on human α WT ENaC in HEK293 cells.

The inward sodium current was measured in the whole cell configuration at a E_h of -100 mV. The control current amplitude of 75.8 ± 4.5 pA. ($n=4$) increased significantly with 5 nM TNF- α . The maximal response to the test compound arises with 20 nM at a current amplitude of 154.5 ± 3.3 pA ($n=4$). The steady state was obtained at this concentration and in the following the ENaC- blocker amiloride was added, which partly blocked the current (54.9 pA).

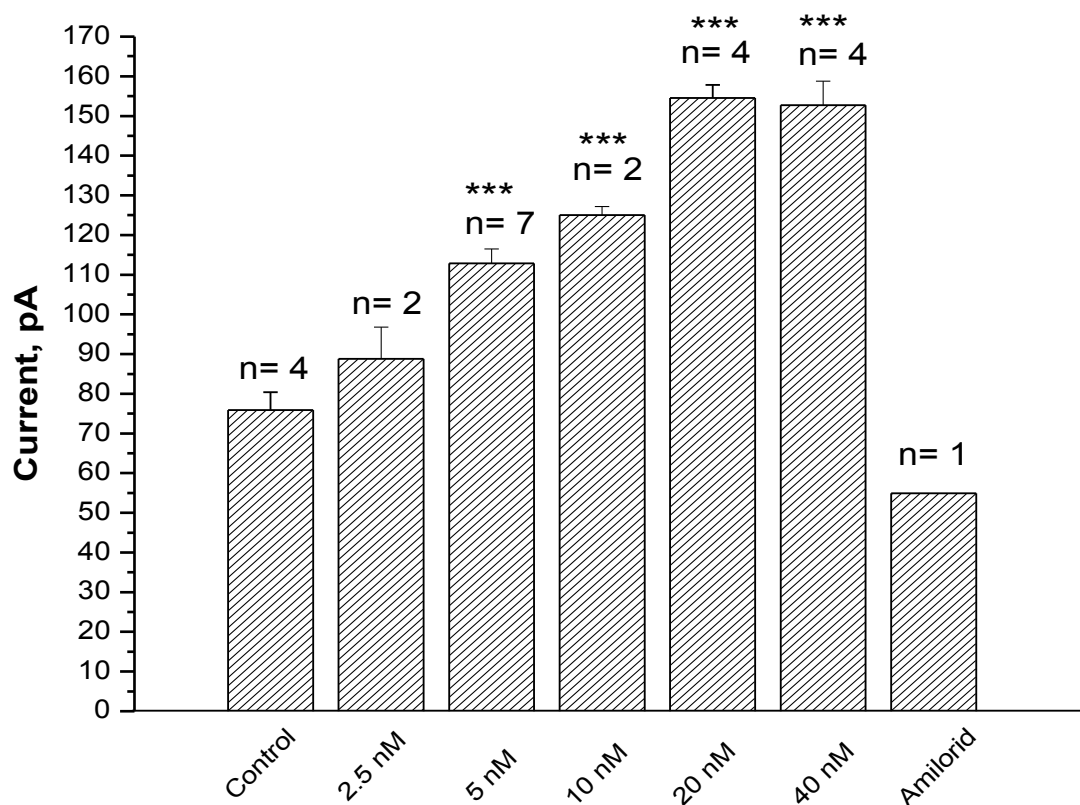


Figure 9: TNF- α induced current through amiloride-sensitive sodium current through $\alpha\beta\gamma$ human WT ENaC in HEK293 cells.

Control current through $\alpha\beta\gamma$ human WT ENaC is 75.8 ± 4.5 pA and steady state is obtained at a concentration of 20 nM. The maximal response to TNF- α is obtained at a current amplitude of 154.5 ± 3.3 pA ($n=4$). Currents were measured in whole cell mode and cells were clamped at a E_h of -100 mV. Mean values $\pm$ S.E. of the inward current. *** significant difference compared to control was determined by paired, two-tailed t-test $P < 0.001$ ($n=2-7$)

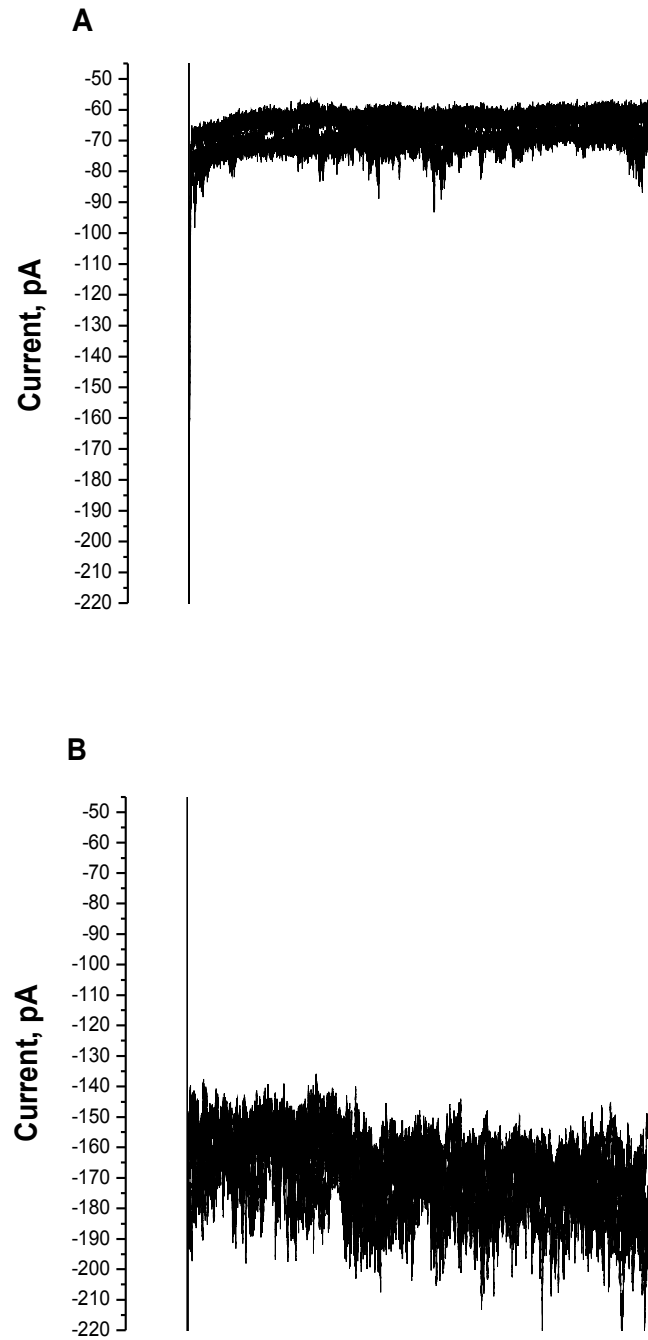


Figure 10: Original whole cell recordings from cells transfected with $\alpha\beta\gamma$ human WT ENaC.

The cells were clamped at E_h of -100 mV. Recordings are shown during control (A) and in presence of 20 nM TNF- α (B).

The dose-response curve and EC_{50} value for α human WT ENaC were fitted and estimated for the currents recorded at -100 mV as shown in Figure 11. The steady state concentration of 20 nM was set as 100 %.

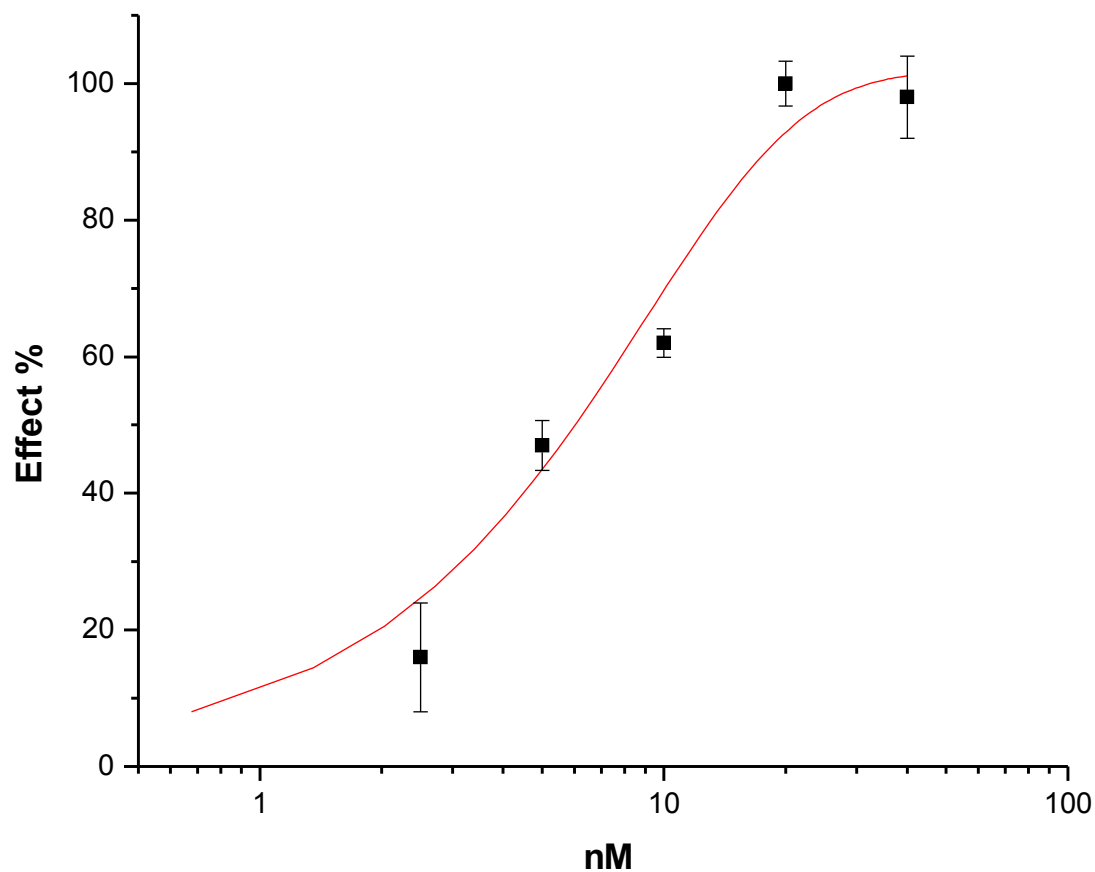


Figure 11: TNF- α dose-response curve for $\alpha\beta\gamma$ human WT ENaC.

The estimated EC_{50} : 6.7 ± 2.1 nM; 20 nM was set as 100 %.

4.2. Concentration-dependent activation of human α N232Q ENaC through TNF- α

Experiments with human α N232Q with $\beta\gamma$ human WT ENaC were conducted to characterize the effect of the N- linked glycosylation sites. Figure 12 shows control currents with an amplitude of 70.8 ± 2.4 pA (n=7). TNF- α was added in concentrations up to 40 nM. The maximal response to TNF- α was achieved at a current level of 100.8 ± 8.8 pA (n=9) with 20 nM (Figure 13). Finally, when steady state effect was obtained the addition of amiloride blocked the sodium current significantly (16.1 ± 14.0 pA ; n=2).

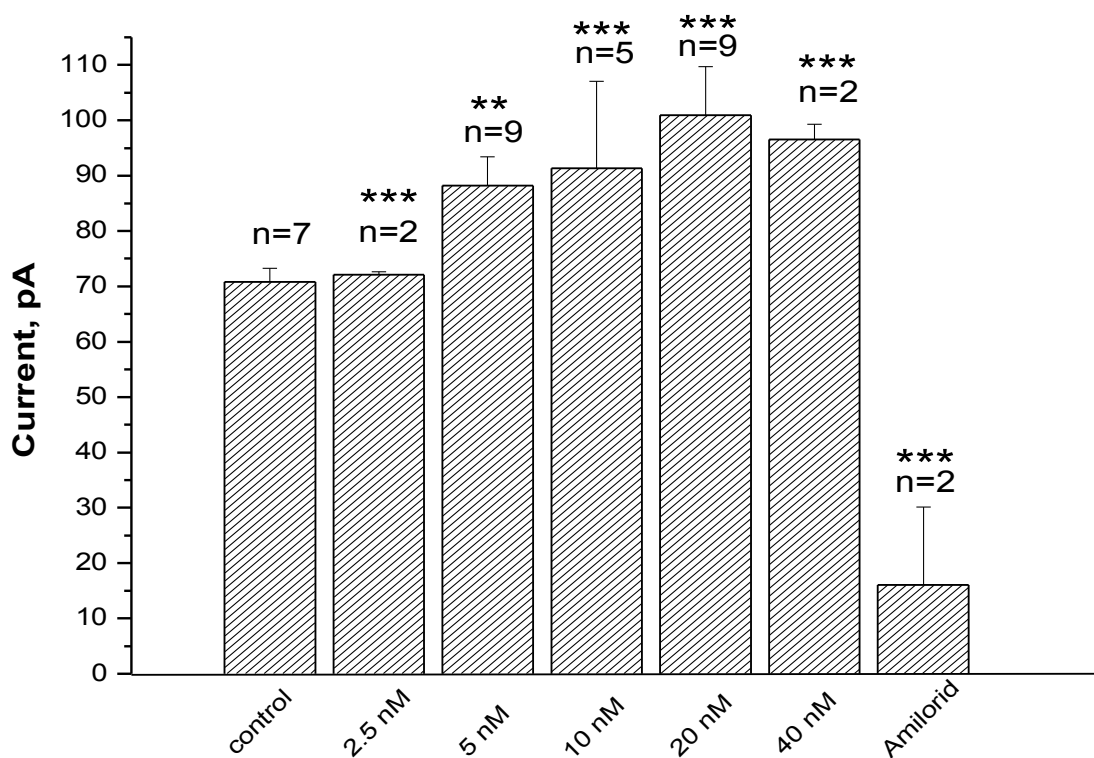


Figure 12: TNF- α induced current through amiloride-sensitive sodium current in HEK293 cells.

Control current through α N232Q $\beta\gamma$ human WT ENaC is 70.83 ± 2.4 pA (n=7) and steady state is obtained at a concentration of 20 nM. Maximal response occurs at 20 nM at an amplitude of 100.9 ± 8.8 pA (n=9). Currents were recorded in whole cell mode and cells were clamped at a E_h -100 mV. Mean values $\pm$ S.E. of the inward current. *** significant difference compared to control was determined by paired, two-tailed t-test $P < 0.001$ (n=2-9); ** < 0.01 (n=5)

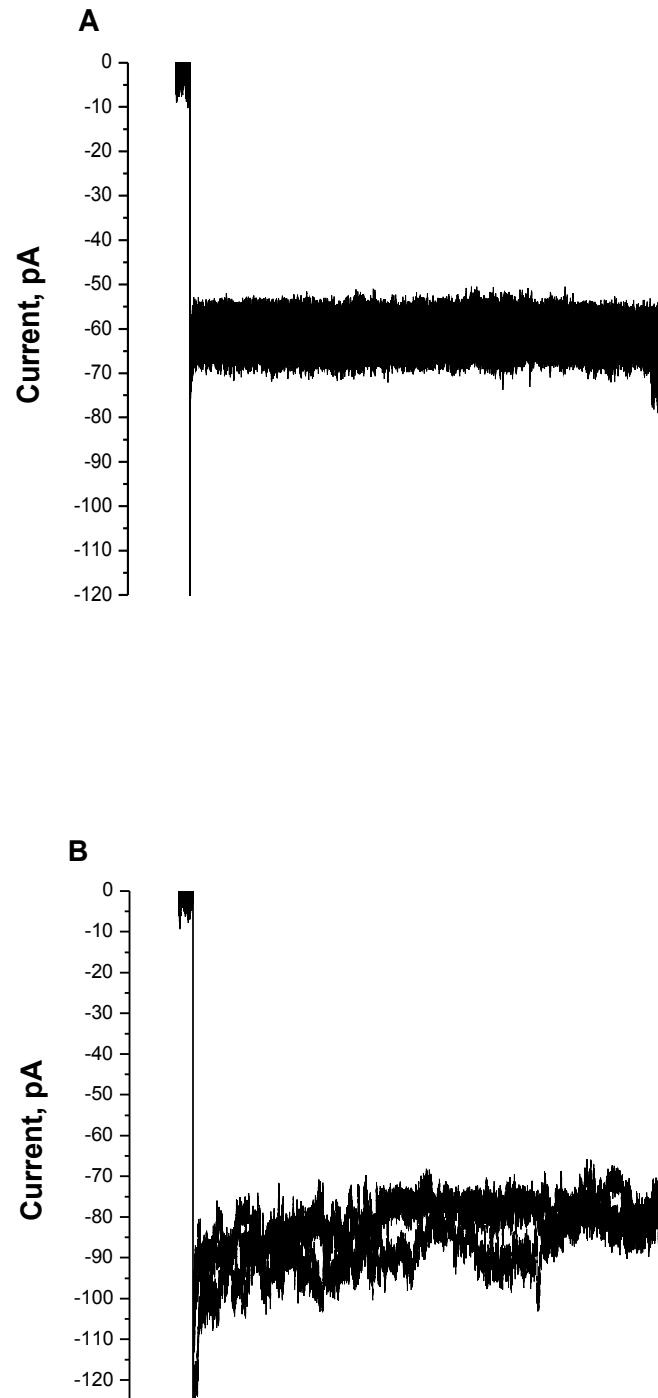


Figure 13: Original whole cell recordings from cells transfected with α N232Q $\beta\gamma$ human WT ENaC. The cells were clamped at E_h of -100 mV. Recordings are shown during control (A) and in presence of 20 nM TNF- α (B).

The dose-response curve and EC_{50} value for $\alpha N232Q$ human ENaC were fitted and estimated for the currents recorded at -100 mV as shown in Figure 14. The steady state concentration of 20 nM was set as 100 %.

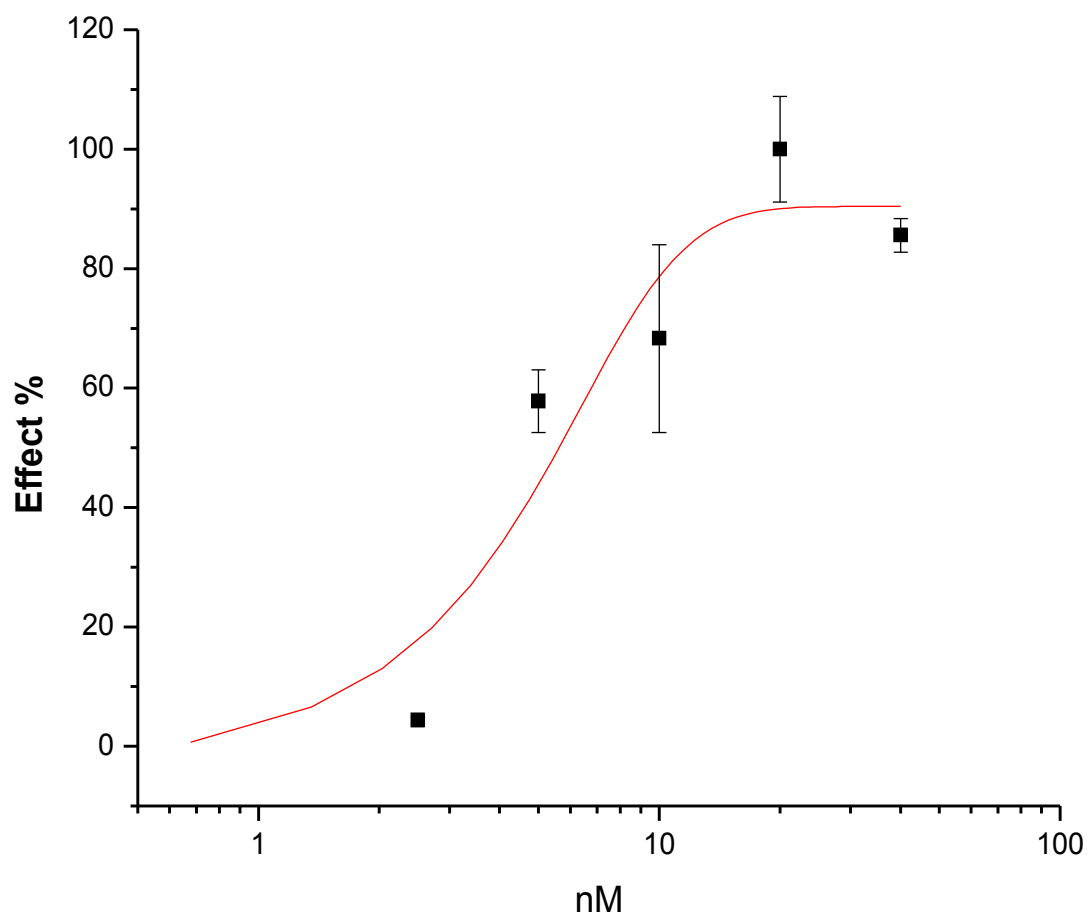


Figure 14: TNF- α dose-response curve for $\alpha N232Q$ $\beta\gamma$ human WT ENaC.

The estimated EC_{50} : 3.6 ± 5.2 nM, 20 nM was set as 100%

4.3. Concentration-dependent activation of human α N511Q ENaC through TNF- α

A further mutation which was generated was the α N511Q mutation. Figure 15 shows the control amplitude at a current of 56.5 ± 4.0 pA ($n=4$). The first significant increase with TNF- α appears with 10 nM. In this case a higher concentration of TNF- α , about 40 nM, was required to achieve steady state though the maximal response was lower than in WT. The maximal response was at a current level of 74.5 ± 6.1 pA ($n=1$). Figure 16 shows the response to 20 nM TNF- α . ENaC was then blocked with amiloride.

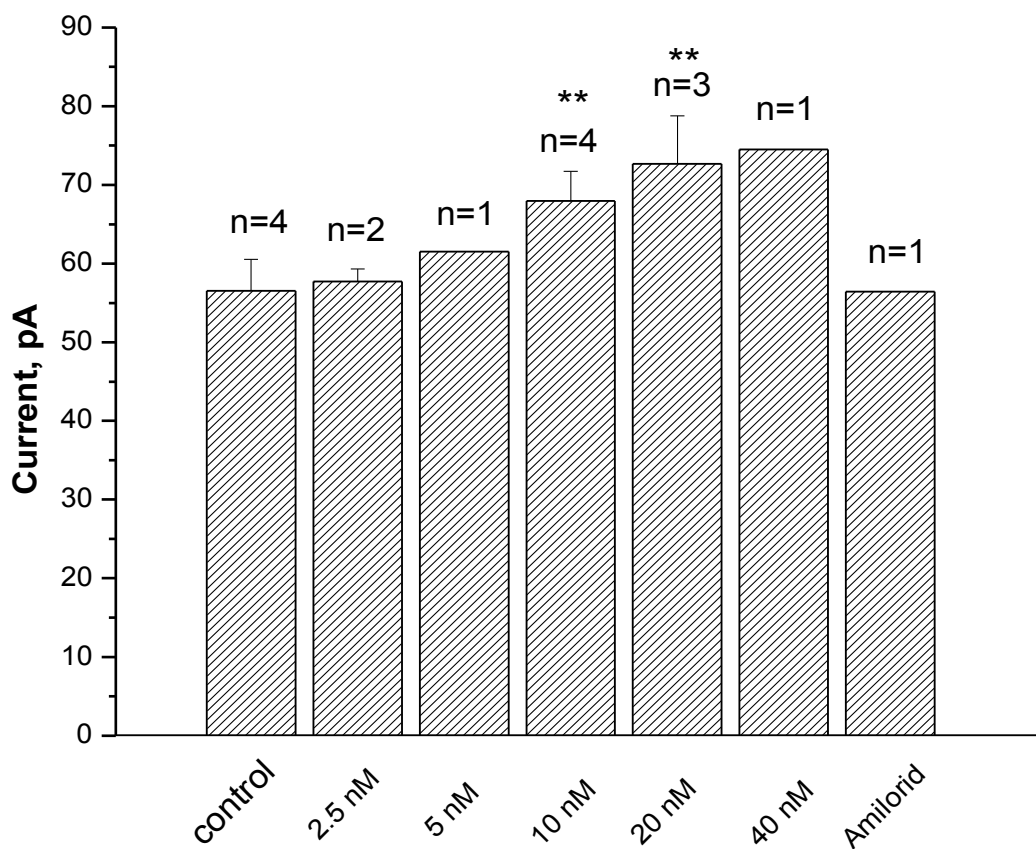


Figure 15: TNF- α induced current through amiloride-sensitive sodium current in HEK293 cells.

Control current through α N511Q $\beta\gamma$ WT hENaC is 56.5 ± 4.0 pA ($n=4$) and steady state is obtained at a concentration of 40 nM with a maximal response at a current level of 74.4 pA. Currents were recorded in whole cell mode and cells were clamped at a E_h -100 mV. Mean values $\pm$ S.E. of the inward current. ** significant difference compared to control was determined by paired, two-tailed t-test $P < 0.01$ ($n=2-4$)

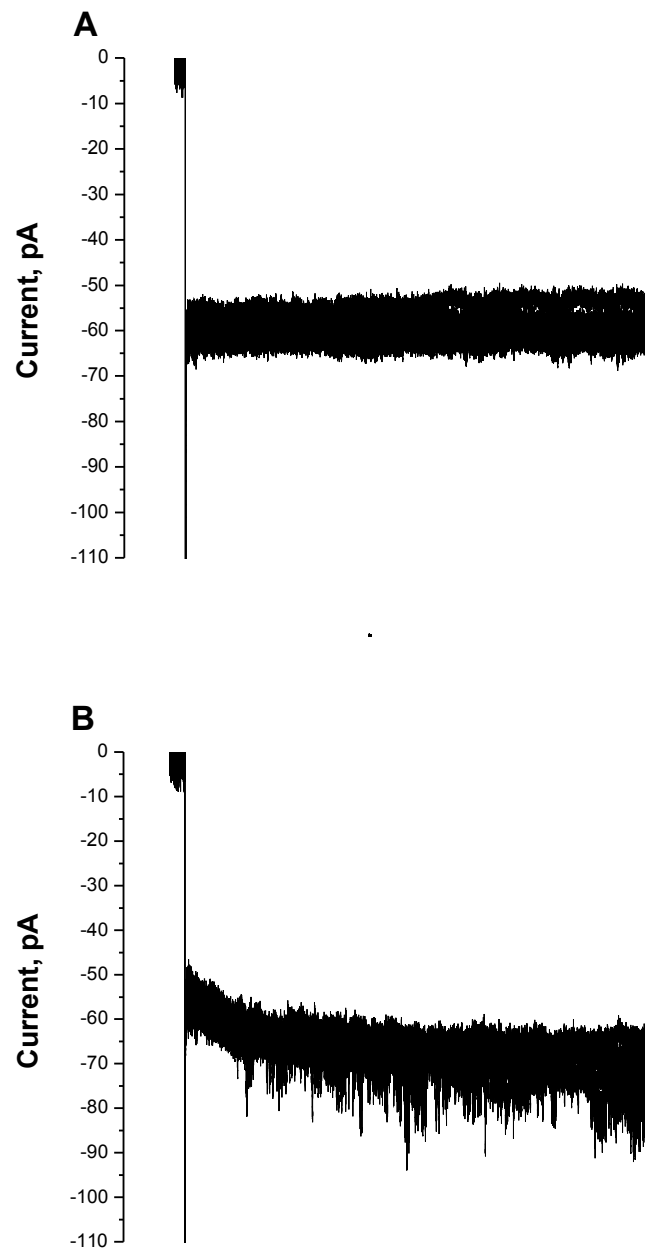


Figure 16: Original whole cell recordings from cells transfected with α N511Q $\beta\gamma$ human WT ENaC. The cells were clamped at E_h of -100 mV. Recordings are shown during control (A) and in presence of 20 nM TNF- α (B).

The dose-response curve and EC_{50} value were fitted and estimated for the currents recorded at -100 mV as shown in Figure 17. The steady state concentration of 40 nM was set as 100 %.

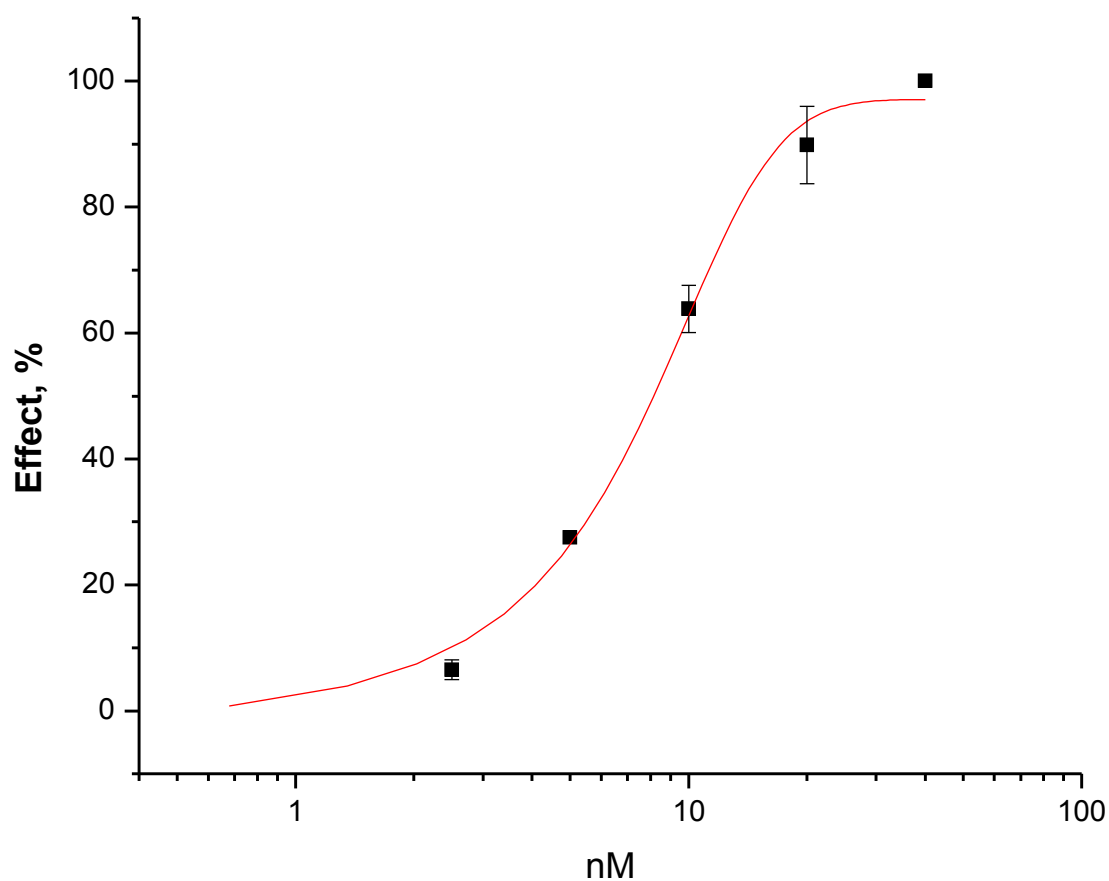


Figure 17: TNF- α dose-response curve for α N511Q $\beta\gamma$ human WT ENaC

The estimated EC_{50} 7.9 ± 0.1 , 40 nM was set as 100%

Summarizing, Table 5 shows all three different EC_{50} values in comparison. The present data shows that a higher amount of TNF- α is needed to have an effect in $\alpha N511Q$ thus this certain glycosylation site seems to be of particular importance for the interaction between TNF- α and ENaC. The EC_{50} of $\alpha N232Q$ is lower than the WT, which allows the assumption that this N-linked glycosylation site is not required for ENaC activation through TNF- α .

Table 5: Potency of TNF- α in human α ENaC WT and mutation in HEK293 cells

ENaC subunit combination in HEK293 cells	EC_{50} (nM)
$\alpha\beta\gamma$ h WT ENaC	6.7 ± 2.0
$\alpha N232Q \beta\gamma$ h WT ENaC	3.6 ± 5.2
$\alpha N511Q \beta\gamma$ h WT ENaC	7.9 ± 0.1

The significant differences of the EC_{50} values ($\alpha N232Q$, $\alpha N511Q$) compared to the EC_{50} value of $\alpha\beta\gamma$ h WT ENaC were determined by unpaired, two-tailed t-test. The differences are not considered to be statistically significant.

5. DISCUSSION

The epithelial sodium channel on the apical surface in the alveolar epithelium plays a key role in the fluid balance in the lung (Matthay et al. 2002). It is expressed along the epithelium of the respiratory system but is especially contained in ATII cells (Berthiaume et al. 1998).

Sodium enters the cell via ENaC or by other cationic channels. It is actively transported from the alveolar space into the interstitium by alveolar epithelial type II cells (Verghese et al. 1999, Berthiaume et al. 1998).

The basolateral located ATP- dependent Na^+/K^+ -ATPase produces an electrochemical gradient by extrusion of sodium from the cells and uptake of potassium into the cells. This mechanism is essential for the vectorial transport of sodium, which is passively followed by water (Matthay et al. 2002). Therefore, this mechanism is essential for normal lung physiology as well as in the pathophysiology.

The pleiotropic cytokine $\text{TNF-}\alpha$, which is produced by macrophages during inflammatory process plays an important dual role in pulmonary edema because of its spatially two distinct binding sites (Yang et al. 2010, Braun et al. 2005). With its receptor binding site located on the basolateral regions of the molecule it contributes to the pathogenesis of pulmonary lung edema. And by contrast, with its lectin-like domain on the tip of the molecule it mediates a protective effect by activating edema reabsorption (Hribar et al. 1999, Braun et al. 2005).

$\text{TNF-}\alpha$ has been reported to increase ALC in rats with pneumonia (Rezaiguia et al. 1997). Furthermore, this positive effect has been studied in rats with intestinal-ischemia-reperfusion (Börjesson et al. 2000). Fukuda et al. (2000) demonstrated the effect in rats and in addition by performance of whole cell patch clamp studies in A549 cells, where an enhanced sodium inward current through $\text{TNF-}\alpha$ could be reported.

Hazemi (2011) has examined the EC_{50} of $\text{TNF-}\alpha$ in A549 cells, which is 8.2 ± 0.1 nM. By contrast, the EC_{50} in HEK293 cells, which were transiently transfected with plasmids coding for $\alpha\beta\gamma$ - WT ENaC is 6.7 ± 2.0 nM. Furthermore, the current which was measured in HEK293 cells is distinct lower than in A549 cells. The maximal effect to $\text{TNF-}\alpha$ in transfected cells achieved a current level of 154.5 ± 3.3 pA, whereby A549 cells exhibit a greater whole cell current at > 1000 pA. There is an obvious difference in the effect of $\text{TNF-}\alpha$ between these different cell lines. Among others A549 cells express ENaC endogenously (Lazrak et al. 2000) and in comparison to

that, the heterologous cell line HEK293 does not show any evidence to express ENaC (Ruffieux-Daidié et al. 2008). The difference may be based on the regulation and trafficking of ENaC. The total number of ENaC at the apical membrane which actively transports sodium is variable in transfected and native cell systems. In transfected cell systems only 1-2 % of ENaC is outside the endoplasmatic reticulum, whereas in native cells (A549) it appears to be much higher (10-30%) . This might be one reason for the difference in the effect of TNF- α between these cell lines (Eaton et al. 2010).

However, TNF- α mediates an effect by increasing the P_o of ENaC by a to date unidentified binding site. Despite that fact, it is suggested that TNF- α interacts with ENaC from the extracellular site upon a protein-carbohydrate interaction by its lectin-like domain (Dulebo et al. 2012). Additionally, it has been shown in whole cell patch clamp technique that the effect of TIP-peptides could solely be observed by adding the test compound into the bath solution (Hazemi et al. 2011).

One of the posttranslational modifications within the ECL include the N-linked glycosylation sites on the outside of the plasma membrane. To date, their functional relevance is not clear since studies indicate that they do not play any role in neither channel assembly, translocation to the membrane nor have any properties in conductance (Benos et al. 1999, Snyder et al. 1994). By contrast, it has been reported for voltage-gated sodium channels that glycosylations have a modulating effect on the conductance properties of these channels (Cronin et al. 2005). The data of this study also demonstrate that control current in WT compared to the mutations of α -subunit are almost equal and the mutation does not seem to affect the conductivity of the α -subunit.

The TIP domain TNF- α has been shown to bind in particular to chitobiose and other carbohydrate motifs in glycoproteins (Marquardt et al. 2007).

Moreover, the role of the N-linked glycosylation sites was studied with TNF- α in A549 after deglycosylation with Peptide-N-glycosidase F (PNGase F) in whole cell and single channel recordings. The experiments clearly demonstrate that the effect of TNF- α was completely lost after treating the cells with PNGase F as shown in Table 6 (Hazemi 2011) .

Table 6: Effect of TNF- α after deglycosylation with PNGase F treatment in A549 cells (Hazemi 2011)

Test compound	Control	Pre-treatment with PNGase F	Untreated cells
TNF- α	25.4 $\pm$ 6.1 pA (n=10)	24.0 $\pm$ 5.9 pA (n= 3)	1073.3 $\pm$ 15.1 pA (n=10)

Shabbir et al. (2013) studied the effect of AP301 in two different cell lines (A549 and HEK293) after deglycosylation with endoglycosidase and showed that AP301 was not able neither to activate ENaC nor increase inward sodium current (Figure 18 A ,B).

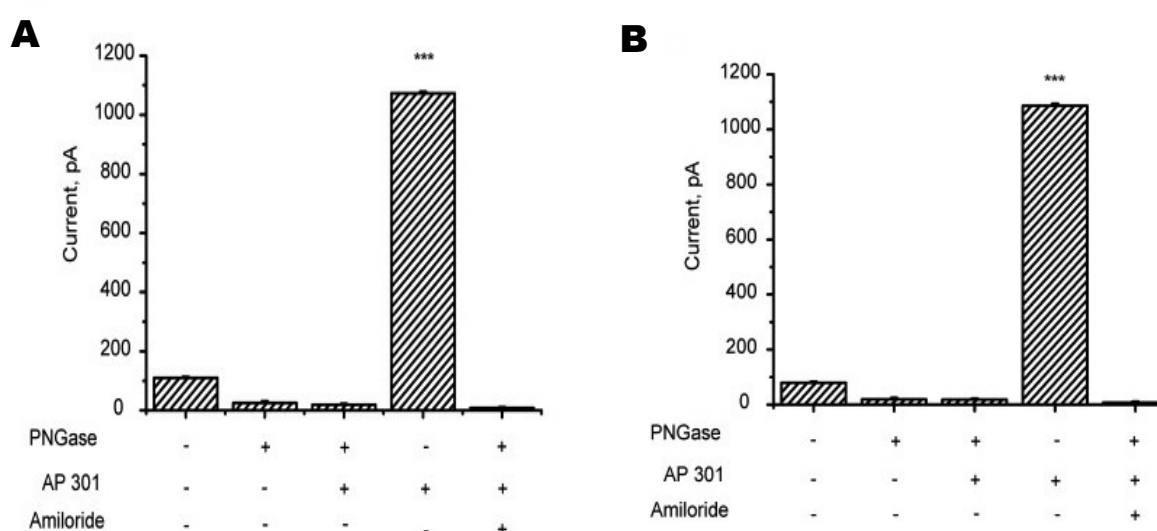


Figure 18: Effect of AP301 before and after treatment with PNGase F in whole cell mode (Shabbir et al. 2013)

(A) AP301 induced amiloride-sensitive sodium current before and after treatment with PNGase F in A549 cells.

(B) AP301 induced amiloride-sensitive sodium current before and after treatment with PNGase F in HEK293 cells.

The data of the current study shows a tendency for a similar effect. After site-directed mutagenesis of α N511Q the EC_{50} is shifted to a higher concentration (7.9 nM $\pm$ 0.1) in comparison to the $\alpha\beta\gamma$ h WT ENaC where the estimated EC_{50} was 6.7 nM $\pm$ 2.0. Although the difference is not significant there is a slight tendency which might indicate the importance of this certain position. This would also support our previous studies with AP301 in A549 cells, where we have recently shown that this certain mutation plays an important role.

In contrast to that the EC_{50} of the mutation $\alpha N232Q$ was 3.6 ± 5.2 . This result shows that this position does not seem to affect the interaction of TNF- α with human α ENaC, just as we have previously shown with AP301 in HEK293 cells.

In summary, it is indicated that the sugar moieties on the cell membrane seem to be crucial for interaction with the lectin-like domain of TNF- α as well as with AP301 the TIP-peptide, which mimics the lectin-like domain of TNF- α .

The obtained results are not sufficient to confirm our previous studies, therefore we need to test further mutated N-linked glycosylation sites and in addition the α ENaC subunit after full deglycosylation in HEK293 cells.

6. CONCLUSION

In conclusion, this study shows that TNF- α is able to increase the sodium current through amiloride-sensitive sodium channel in a transiently transfected cell system, whereby α N511Q seems to be an important position for activation.

All in all, this data gives us a hint that the binding of TNF- α requires precedent interaction with a glycosylated extracellular loop before binding to a specific unknown binding site of ENaC on the carboxy terminal (Czikora et al. 2014, Shabbir et al. 2013). But for a precise conclusion TNF- α needs to be tested again in addition after full deglycosylation in HEK293 cells in order to confirm our previous data and assumption.

7. APPENDIX

7.1. List of abbreviations

A549	Adenocarcinomic human alveolar basal epithelial cells
ALC	Alveolar liquid clearance
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
AT I	Alveolar cell type I
AT II	Alveolar cell type II
CNG	Cyclic nucleotide-gated channels
CFTR	Cystic fibrosis transmembrane conductance regulator
DMEM- F-12	Dulbecco' s modified Eagle' s medium/ nutrient mixture F12-Ham
DNA	Desoxyribonucleic acid
EC ₅₀	half maximal effective concentration
ECL	Extracellular loop
Eh	Holding potential
ENaC	Epithelial sodium channel
FBS	Fetal bovine serum
hENaC	human epithelial sodium channel
HEK 293 cells	Human embryonic kidney 293 cells
HSC	High- selective sodium channel
Nedd4-2	Neural precursor cell expressed developmentally down-regulated 4 protein
NSC	Non-selective sodium channel
PBS	Phosphate buffered saline
PNGase F	Peptide- N-glycosidase F
Po	Open probability
rENaC	rat epithelial sodium channel
RT	Room temperature
TIP	Lectin-like domain
TNF- α , mTNF- α	Tumor necrosis factor-alpha , mouse TNF- α
WT	Wild type

7.2. List of tables

Table 1: Cell culture reagents

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7.3. List of figures

Figure 1: Normal alveolous on the left hand and injured alveolous on the right hand

Figure 2: Schematic diagram of the distal pulmonary epithelium with different alveolar type cells

Figure 3: Structure of epithelial sodium channel

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Figure 5: Dichotomal role of TNF- α

Figure 6a: Structure of TNF- α at 2.6 Å resolution

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Figure 7: Cell attached and whole cell mode in patch clamping

Figure 8: Membrane topology of ratENaC

Figure 9: TNF- α induced current through amiloride-sensitive sodium current through $\alpha\beta\gamma$ human WT ENaC in HEK293 cells

Figure 10: Original whole cell recordings of cells transfected with $\alpha\beta\gamma$ human WT ENaC

Figure 11: Dose-response curve of TNF- α in $\alpha\beta\gamma$ human WT ENaC

Figure 12: TNF- α induced current through amiloride-sensitive sodium current through α N232Q $\beta\gamma$ human WT ENaC in HEK293 cells

Figure 13: Original whole cell recordings of cells transfected with α N232Q $\beta\gamma$ human WT ENaC

Figure 14: Dose-response curve TNF- α in α 232Q $\beta\gamma$ human WT ENaC

Figure 15: TNF- α induced current through amiloride-sensitive sodium current through α N511Q $\beta\gamma$ human WT ENaC in HEK293 cells

Figure 16: Original whole cell recordings of cells transfected with α N511Q $\beta\gamma$ human WT ENaC

Figure 17: Dose-response curve of TNF- α in α N511Q $\beta\gamma$ human WT ENaC

Figure 18: Effect of AP301 before and after treatment with PNGase F in whole cell mode

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