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Titel der Diplomarbeit / Title of the Diploma Thesis

„Evaluation of the effect of synthetic TNF lectin like domain derived peptide AP318 (TIP peptide) on Pseudohypoaldosteronism type IB (PHA 1B) α stop codon mutants of the epithelial sodium channel (ENaC)"

verfasst von / submitted by

Florian Daniel Vogel

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Contents

Abstract

ENaC is a major regulating factor regarding electrolyte balance, blood pressure and alveolar fluid clearance by reabsorbing Na^+ through highly resistant epithelial membranes. Literature tells that in total there are more than 50 single point mutations in any of the three subunits ENaC is assembled of, namely α-, β- and γ-subunit, which can lead to a severe life threatening, salt wasting disease called Pseudohypoaldosteronism type IB (PHA1B). From electrophysiological experiments, conducted at the Department of Pharmacology and Toxicology of the University of Vienna, it was already known that four PHA1B α-stop-codon mutants show lower basal sodium current compared to the wild type, which can be rescued by the synthetic cyclic TIP peptide AP318. The membranal surface expression of those mutants was ill defined though and the effect of AP318 on the integral membrane expression of ENaC wild type (WT) compared to the stop-codon-mutants was uncharacterized. The aim of this thesis was to investigate the effect of AP318 on the cell surface expression of four α-stop-codon ENaC mutants of transiently transfected, ectopically expressing HEK cells, in comparison with ENaC WT. Therefore, HEK cells were transfected with ENaC WT- or mutant DNA. Membrane proteins were isolated after AP318 treatment through NHS-biotin dependent protein purification and separated on a SDS-polyacrylamide gel. Protein bands were detected with anti-α-ENaC antibodies. Loading control was performed with β-Actin. The proposition of this thesis is, that AP318 enhances membrane expression of ENaC WT and α-stop-codon mutants with a maximum effect at 30 minutes of treatment, with the exception of the mutant αC63X, which was not detected at all.

Kurzzusammenfassung

ENaC ist ein wichtiger Regulierungsfaktor für Blutdruck, Alveolarflüssigkeitsbilanz und Elektrolytgleichgewicht, indem er Na^+ durch schwer durchlässige Epithelmembranen reabsorbiert. Aus der Literatur geht hervor, dass es in jeder der drei Untereinheiten, aus denen ENaC aufgebaut ist, namentlich α-, β- und γ-Untereinheit, insgesamt mehr als 50 Einzelpunktmutationen gibt, die zu einer schweren, lebensbedrohlichen, mit Salzverlust einhergehenden Krankheit führen können, die als Pseudohypoaldosteronismus Typ IB (PHA1B) bezeichnet wird (Hanukoglu and Hanukoglu, 2016). Aus elektrophysiologischen Experimenten des Departments „Pharmakologie und Toxikologie“ der Universität Wien geht hervor, dass vier PHA1B-α-Stop-Codon-Mutanten im Vergleich zum Wildtyp einen niedrigeren basalen Natriumstrom aufweisen, der durch das synthetische cyclische Peptid AP318 auf ENaC WT Niveau angehoben werden kann. Die Membranoberflächenexpression dieser Mutanten ist bis dato undefiniert, und die Wirkung von AP318 auf die Expression von ENaC Wildtyp (WT) im Vergleich zu vier Stopcodonmutanten ist uncharakterisiert. Das Ziel dieser Diplomarbeit war die Untersuchung der AP318 Wirkung auf die Oberflächenexpression von vier α-Stop-Codon ENaC-Mutanten transient transfizierter, ektopisch exprimierender HEK-Zellen, in Bezug auf ENaC WT. Daher wurden HEK-Zellen mit ENaC WT oder mutierter DNA transfiziert. Membranproteine wurden nach AP318-Behandlung durch NHS-Biotin-abhängige Proteinaufreinigung isoliert und auf einem SDS-Polyacrylamidgel aufgetrennt. Proteinbanden wurden mit anti-α-ENaC-Antikörpern nachgewiesen. Ladekontrolle wurde mit β-Actin durchgeführt. Die These dieser Diplomarbeit ist, dass AP318 die Membranexpression von ENaC WT- und α-Stop-Codon ENaC-Mutanten mit maximaler Wirkung nach 30-minütiger Behandlung erhöht, mit Ausnahme der Mutation αC63X, welche in Western Blot Experimenten nicht nachgewiesen werden konnte.

Prehistory

Apeptico, a privately held biotechnology company developing peptide drugs located in Vienna invented peptide drugs derived from the lectin-like domain of TNF-α. One of these, Solnatide (aka AP301), is being developed for the treatment of oedematous respiratory failure in patients suffering from lung infection, lung injury and lung transplantation (*Press Release Apeptico*, 2011). This compound showed significant results in the experimental lung transplantation model to prevent ischemia reperfusion injury (Press Release Apeptico, 2010). Two Phase II clinical trials with orally inhaled peptides for treatment of pulmonary permeability oedema and acute respiratory distress syndrome (ARDS) and for treatment of primary graft dysfunction (PGD) following lung transplantation, were successfully completed by Apeptico (Press Release 2016). Besides ARDS and PGD, Solnatide has already gained orphan drug designation for the treatment of high altitude pulmonary edema (HAPE) and pseudohypoaldosteronism type 1b (Press Release Apeptico, 2016).

A recessively handed on genetic “Loss of function ENaC” is associated with symptoms of pseudohyperaldosteronism type IB, a severe, life threatening, salt wasting disease (Riepe, 2009). Apeptico, in collaboration with University of Vienna, research group of Prof. Dr. Lemmens-Gruber, has conducted numerous ground-breaking studies proving the effect of AP301 on ENaC (Shabbir *et al.*, 2013, 2015; Willam et al., 2017b). They have been testing the effect of Solnatide AP301 on ENaC WT and various ENaC mutations, which lead to manifestation of the phenotype disease pseudohypoaldosteronism type IB. Back in 2008, Apeptico also developed a bunch of chemical derivates of Solnatide, whereas the compound AP318 emerged as the most promising candidate for the treatment of ENaC loss of function, besides Solnatide, respectively. Previous electrophysiological data from the Department of Pharmacology and Toxicology of University of Vienna showed lower EC_{50} of AP318, on ENaC WT and PHAIB related mutants than AP301, regarding sodium currents (Willam *et al.*, 2017a). It was not fully characterized, whether this is the result of an increased open probability/ prolonged open conformation state, an enhanced membranal ENaC expression or a combination of both effects. It was to investigate, whether cell signalling

experiments can support the electrophysiological data. AP318 is a very good candidate for further drug development regarding the treatment of PHA1B patients.

Aim of the Master Thesis

The aim of this work was to evaluate the effect of synthetic TNF lectin like domain derived peptide AP318 (TIP-peptide) on Pseudohypoaldosteronism Type IB (PHA IB) α-stop-codon mutants of the epithelial sodium channel (ENaC). It was to investigate, whether the treatment of transiently transfected, ectopically ENaC-expressing HEK-293 cells with the TIP-peptide AP318 alters cell surface expression of ENaC. The data our group already won through electrophysiological experiments (patch clamp), show enhanced activity of ENaC wild type (WT) as well as PHA1B related ENaC stop-codon mutants, in presence of AP318. Even though, cell surface expression does not linearly correlate with channel activity, the enhanced Na^+ currents induced by AP318, might be partially due to the increase of functional membranal ENaC.

Epithelial Sodium Channel – Function

ENaC, often described as „amiloride sensitive sodium channel“ is a non-voltage-gated sodium ion channel which belongs, as well as the structural related “acid sensing ion channel“ (ASIC) to the ENaC/degenerin ion channel family. It is located at the apical site of epithelial cells and is highly expressed in tissues such as the kidneys, lungs, colon and the salivary glands amongst others, where it is responsible for the reabsorption of sodium through highly resistant barriers. In the distal convoluted tubule of the kidneys it is a major factor in regulating blood pressure and electrolyte balance, by reabsorbing sodium ions from the primary urine back into the blood vessels. On the basolateral site of the cell a Na^+/K^+ antiporter is located to maintain the sodium gradient. It pumps out sodium into the interstitial fluid in exchange for potassium ions (3 Na^+ leave the cell, 2 K^+ enter it) (Figure 1). The intracellular gained potassium ions are later excreted in a separate event by the renal outer medullary potassium channel (ROMK), whereas ENaC is the driving force for that process. In the lungs, ENaC is responsible for regulating

alveolar liquid clearance, hence lung fluid homeostasis. In the colon, sodium reabsorption is also accomplished by ENaC and in contrast to the kidneys, does not diminish with age (Greig *et al.*, 2003), which is why it might have a big stake in electrolyte homeostasis in elderly. Considering all this, ENaC is responsible for the regulation of electrolyte balance, fluid homeostasis, blood pressure and alveolar liquid clearance, whereas it is mainly regulated by mineralocorticoids.

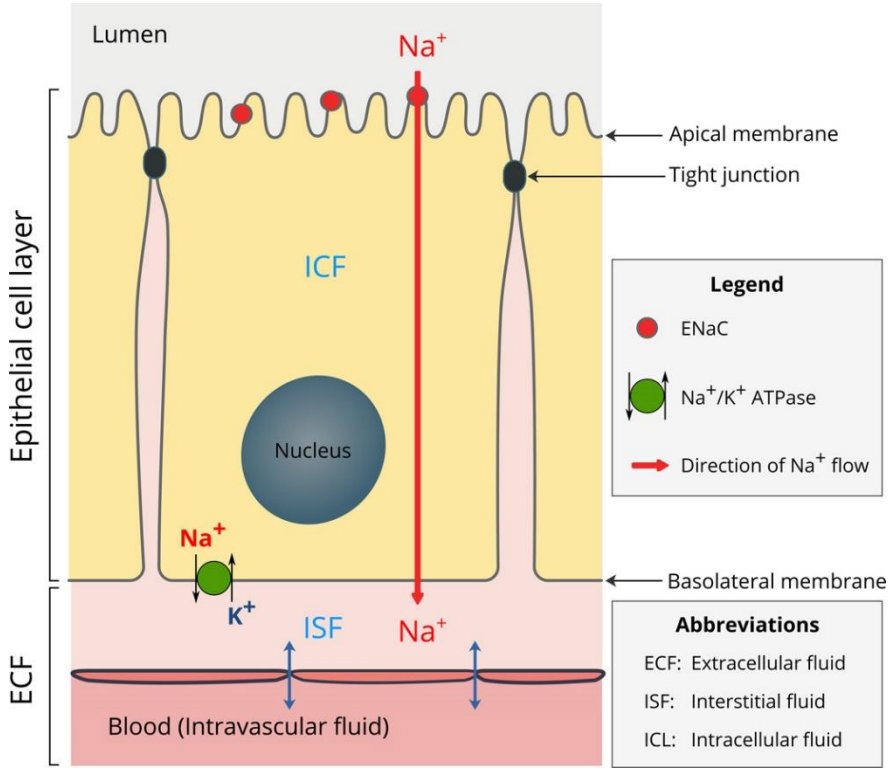

Figure 1 Na+ reabsorption in epithelial cells by ENaC (Hanukoglu and Hanukoglu, 2016b)

Pseudohypoaldosteronism Type IB

The importance of efficient ENaC dependent sodium reabsorption becomes explicit, when giving thought to the consequences that are associated with an ENaC dysfunction. Many known diseases - including Pseudohypoaldosteronism type IB, Liddle syndrome or cystic fibrosis - are associated with dysregulation of the genes which encode for ENaC or dysregulation of ENaC's function and/or expression and/or cell trafficking (Bonny and Hummler, 2000).

Cheek and Perry first described the syndrome of PHA back in 1958 (Cheek and Perry, 1958). The Hanukoglu brothers Aaron and Israel later observed with their

team the existence of two clinically and genetically distinct PHA forms with either renal (PHA1A) or multiple target organ defects (PHA1B) (Hanukoglu, 1991; Hanukoglu *et al.*, 1997).

It is differentiated between two forms of Pseudohypoaldosteronism Type 1 – A and B. Type A is autosomal dominantly handed on loss of function mutation of the mineral corticoid receptor (MCR) and affects renal sodium transport (Geller *et al.*, 1998). Type 1A is the milder form which may be sporadic and remits with age. Type 1B is an autosomal recessively handed on multi organic disease (systemic PHA1), causing severe life threatening, salt wasting states, hyponatremia, hyperkalemia, dehydration and failure to thrive (Hanukoglu and Hanukoglu, 1991). PHA1B implies a lifelong sodium chloride supplement and does not improve with age (Zennaro *et al*., 2012). Loss of function mutations affecting the genes which are encoding for any of the three ENaC subunits (α,β,γ but not δ) can result in the manifestation of PHA1B, while an involvement of a MCR mutation could be excluded (Hanukoglu and Hanukoglu, 2016a).

Physiologically, an electrolyte imbalance or lowered blood pressure initiate the secretion of the hormone Aldosterone in the adrenal cortex of the kidneys via the Renin-Angiotensin-Aldosterone-System (Aguilera *et al*., 1978). Aldosterone is responsible for increasing ENaC expression and enhancing the transport of ENaC from a subapical intracellular storage into the cell membrane via effector proteins, therefore also increases ENaC's number and overall activity (Soundararajan *et al*., 2011). However, ENaC mutants show severe Aldosterone unresponsiveness, which is why ENaC function is affected in multiple tissues in PHA1B patients: In the kidneys the salt reabsorption is poorly taking place - manifesting a constantly life threatening, salt wasting disease (PHA1B) accompanied by dehydration, low blood pressure and inadequate fluid ion balance (hyponatremia, hyperkalemia, acidosis), which are major risks for developing heart arrhythmias and as a consequence, heart attacks. For that reason, babies suffering from PHA1B die very early on, if not treated - whereas treatment is only symptomatic so far in form of acute sodium chloride supplementation. In the lungs, ENaC is responsible for regulating alveolar liquid clearance, and thus lung fluid homeostasis. PHA1B related loss of function ENaC leads to higher incidence of respiratory tract infections, wheezing and persistent rhinorrhea, whereas some patients show

increased liquid volume in airway epithelia. No long term solution for the treatment of PHA1B related symptoms is available on the market yet. AP301 (Solnatide) has gained an orphan drug designation for the treatment of Pseudohypoaldosteronism type IB, acute lung injury, acute respiratory distress syndrome and pulmonary oedema, caused by lung infection or lung transplantation and is being tested in clinical Phase IIB for pulmonary permeability oedema and acute respiratory distress syndrome (ARDS) (Press Release Apeptico, 2016). Due to the positive effect of TIP peptides on ENaC loss of function. In the future, AP318, a derivate of Solnatide, hopefully provides PHA1B patients with the first ever causative treatment, in contrast to the symptomatic treatment, which is available yet and gives them hope to mitigate their symptoms.

Amiloride

As ENaC is often described as amiloride sensitive sodium channel, it is generally known that amiloride blocks ENaC directly by obstructing the pore very potently with an IC_{50} of 0.1µM when consistent of α,β,γ or δ,β,γ (Kellenberger and Schild, 2002).

The interaction between amiloride and ENaC is commonly used for K^+ retaining high blood pressure treatment as a single therapeutic or, more frequently, combined with other K^+- depleting diuretics such as thiazides or loop diuretics like furosemide.

Amiloride most likely binds to the residue of the second transmembranal segment around α-Ser583, β-Gly525 and γ-Gly542 (Kashlan et al., 2005). A team from the University of Pittsburgh, Pennsylvania, discovered a great decrease of amiloride dependent sodium block when mutating the glycines in the β- or γ- subunit but only a moderate one after mutating α-Ser583 (Kashlan et al., 2005).

ENaC Gen locus

The Genloci for the ENaC encoding genes are seated on the short arm of the 12th chromosome for the α-subunit, on the short arm of the 16th chromosome for the β- and γ-subunit and on the short arm of the first chromosome for the δ- subunit. (Hanukoglu and Hanukoglu, 2016a)

SCNN1A 12p13

SCNN1B 16p12.2-p12.1

SCNN1D 1p36.33

SCNN1G 16p12.2

While no mutation in the δ-subunit causing PHA1B has been found yet, stop codon mutations in either the SCNN1A, SCNN1B and SCNN1G gene encoding for the α- , β- and γ-subunits are associated with PHA1B related symptoms (Bonny *et al.*, 2002; Edelheit *et al.*, 2005; Belot *et al.*, 2008). Those mutations lead to shorter versions of the original membrane protein. Depending on the position at which the mutation occurs, mutants lack parts of the carboxy terminus to elements of the TM2 region or even most segments of the extracellular loop. As a consequence, those proteins show altered protein expression, cell trafficking, whole cell currents and membrane presence. Due to the stop mutation and lacking C-terminus, ENaC is unresponsive even to the compensatory high aldosterone levels in contrast to ENaC wild type (Hanukoglu, 1991).

At the moment the Ensembl genome database includes 540 ENaC superfamily homologous genes - 188 of them encoding for either α-, β-, or γ-subunit of ENaC. ENaC subunits are found in vertebrates but not in bacteria, fungi nor plants (Hanukoglu and Hanukoglu, 2016).

To study orthologues of ENaC between species does contribute to our understanding of the subunits function and organization, gives insight into the

folding and structure of the native channel. Especially looking at conservative regions between species gives important insight in alternatives of ENaC expression, if a species lacks ENaC genes (Hanukoglu and Hanukoglu, 2016a).

Structure of the epithelial sodium channel (ENaC)

General

Due to the structural clarification of the homologous ASIC and the recently received cryo-EM structure of ENaC, it is known that ENaC assembles in the form of a homologous heterotrimer (Kashlan and Kleyman, 2011; Noreng *et al.*, 2018) in the apical membranes of cells of different tissues, such as the distal tubule in the nephron, the airway, sweat glands and colon (Planès and Caughey, 2007; Petryszak *et al.*, 2014). ENaC's heterotrimer is consistent of an α- (or delta subunit), a β- and a γ-subunit. α/β/γ heterotrimers are persistent in the kidneys, the sweat glands or the colon, - whereas δ/β/γ formation is observed in the brain, the ovaries and testes (Hanukoglu and Hanukoglu, 2016a; Petryszak *et al.*, 2017) and seems to be able to lead higher sodium currents in oocytes – 2 fold higher single channel activity, 11 fold higher amiloride sensitive Na^+ currents (Haerteis *et al.*, 2009).

The α- or δ-subunits are considered to be the pore forming subunits - without them there is very little sodium current observed – while actually a triad of the three TM2 segments forms the channel's pore. β- and γ-subunits are considered to be important for ENaC activation, gating, stability as well as the regulation of sodium currents passing the channel (Hamm *et al*., 2010). Literature tells that a single point mutation in any of the three subunits can result in PHA1B related symptoms (Hanukoglu and Hanukoglu, 2016a). Structurally, the subunits are closely related based on their primary sequence with each composed of short intracellular N- and C-termini, two transmembrane segments (TM1 and TM2) and a large extracellular loop. The extracellular loop is called the ectodomain of ENaC, being the part of the channel that is going extracellular from TM1 to TM2. All subunits share an approximately equal length, whilst α is composed of 669 amino acids (AS), β of 640 AS, γ of 649 AS and delta of 638 AS (Table 1). The ectodomain is by far the biggest domain of ENaC, taking more of 2/3 of the channel's size (Figure 3). Within

the α- and γ-subunit, the extracellular loop contains inhibitory tracks which can alter the channel's activity by serving as cleavage sites for proteases. The C- terminus includes the very important PY motif. It is called like that due to its richness on proline (P) and tyrosine (Y) and plays a major role in replenishment and degradation of the channel. A mutation in the PY motif can lead to Liddle syndrome, a hereditary form of high blood pressure. (Kashlan and Kleyman, 2011)

	Cytoplasmic N-ter	TM1	Extracellular	TM2	Cytoplasmic C-ter
Alpha	1–84	85–106	107–543	544–575	576–669
Beta	1–49	50–70	71–514	515–546	547–640
Gamma	1–53	54–79	80–523	524–555	556–649
Delta	1–87	88–107	108–520	521–552	553–638

Table 1 Composition of ENaC subunits (Hanukoglu and Hanukoglu, 2016)

N-terminus

The N-terminus is considered to contribute to ENaC assembling, gating as well as endocytosis of the channels (Adams *et al*., 1997; Horisberger, 1998; Gründer *et al.*, 1999). The deletion of longer segments of the N-terminus leads to significant decrease in channel activity (Bachhuber et al., 2005), whereas Chalfant et al. found that deletion of the amino acids 2-67 manifests a longer half life time of channels, suggesting an endocytotic motif within the N-terminus (Chalfant et al., 1999).

TM1

As well as the TM2, the TM1 is anchored into the membrane according to the positive inside rule (von Heijne, 1992), including 2-3 positively charged arginine residues at the cytoplasmic end of the TM1, enabling the channel to interact with cytoplasmic lipids and proteins (Hanukoglu and Hanukoglu, 2016a). Furthermore, aromatic amino acids such as tryptophan and tyrosine were found to be predominant in the membrane water interface, thus enabling stronger anchoring (Pogozheva et al., 2014).

Ectodomain

In detail, derived from the solved structure of the related ASIC and the recently published Cryo-EM structure, it is generally accepted that ENaC's extracellular domain is highly ordered being evocative of a humans hand regarding its form (Jasti *et al.*, 2007; Noreng *et al.*, 2018). The defined subdomains are the finger, knuckle, thumb, palm, wrist and β-ball. The finger domains are located at the proximal outer site of the ectodomain and are likely to differ from the ASIC and even between ENaC's subunits. Functionally, they are accessible for proteases (Caldwell et al., 2004; Rossier and Stutts, 2009; Ray and Kleyman, 2015). According to literature the α-subunit contains two furin cleavage sites from Arg 205 to Arg 231, which encompass an inhibitory tract. In the γ-subunit a furin and a prostasin cleavage site are persistent, each one flanking an inhibitory track (Figure 2). Mutated furin sites in the α-subunit lead to 90% decrease in sodium currents in furin-deficient Chinese hamster ovary cells (Carattino et al., 2008). Nevertheless, stepwise furin and prostasin dependent cleavage in the α-, as well as subsequently the γ-subunit, is necessary for a full activation, thus high sodium currents (Carattino et al., 2008). Despite furin and prostasin being the best investigated proteases to activate ENaC, also other serine proteases such as trypsin, and serpin, plasmin and elastase show activation of the channel. In contrast, the β-subunit lacks protease cleavage sites, but is characterized by additional N-linked glycosylation sites and an additional pair of cysteines, compared to α- and γ-subunits, respectively (Figure 2) (Kleyman et al., 2009). Within ENaC family members there are 16 cysteine residues highly conserved most likely to form 8 disulfide bonds (Hanukoglu and Hanukoglu, 2016a).

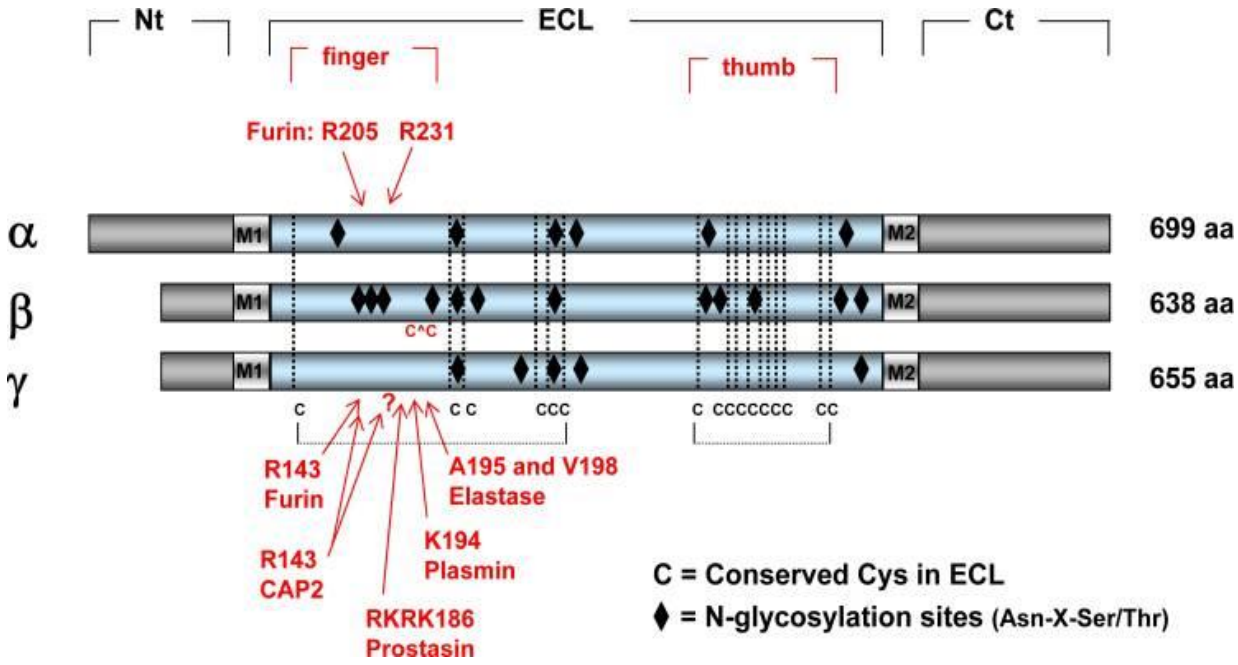

Figure 2 Linear models of ENaC subunits. α- and γ-subunit contain cleavage sites, whilst the β-subunit includes a unique pair of cysteine residues as well as additional N- glycosylation sites (Kleyman et al., 2009)

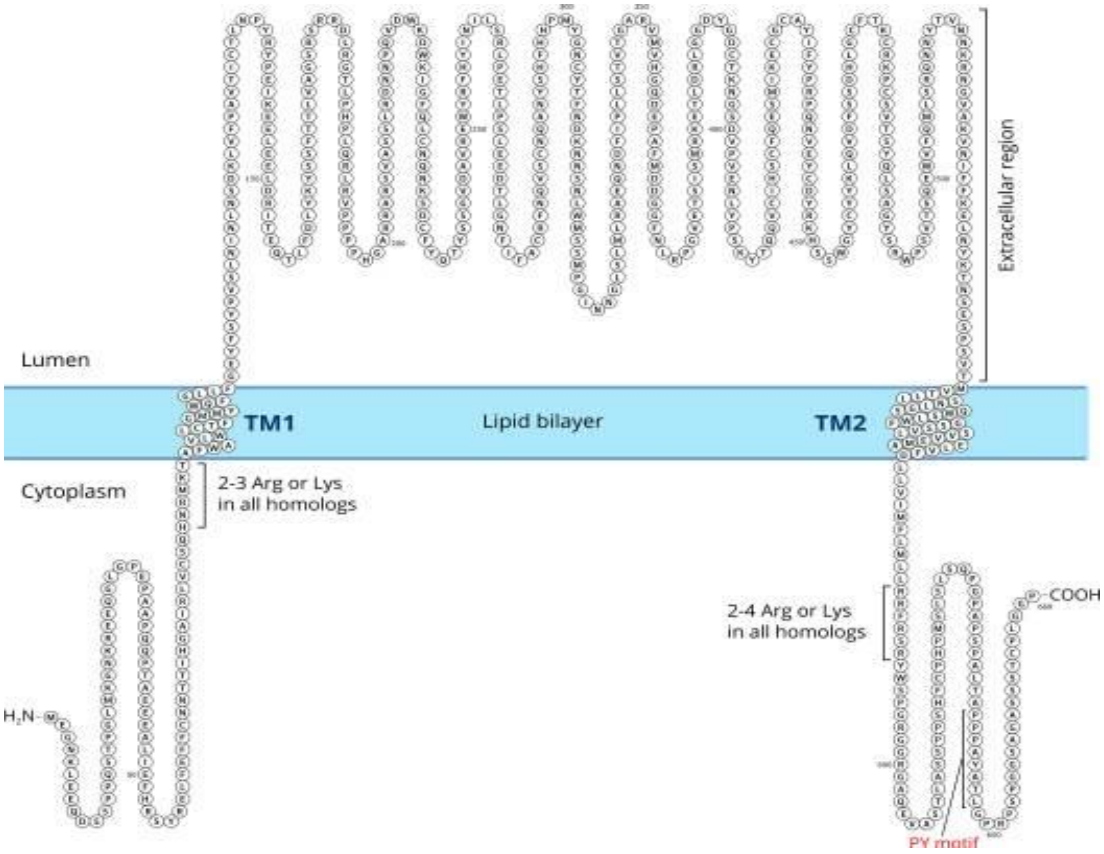

Figure 3 Schematic illustration of the transmembrane localization of an ENaC subunit (Hanukoglu and Hanukoglu, 2016)

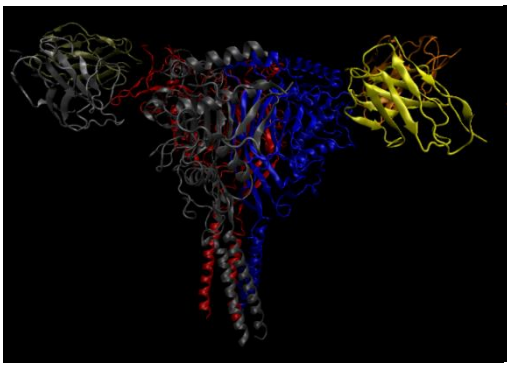

Figure 4 Model of ENaC according to cryo-EM structure of α,ß,γ-ENaC from Noreng et al. (2018), front view

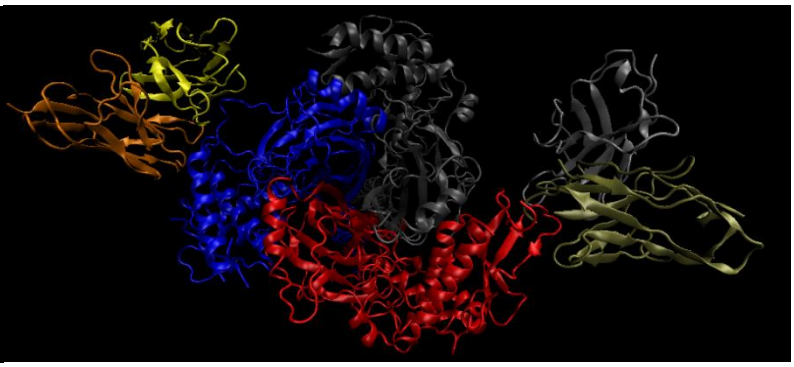

Figure 5 Model of ENaC according to cryo- EM structure of α,ß,γ-ENaC from Noreng et al. (2018), top view

TM2

The TM2 domain plays a major role in the channels gating with the region encompassing an LLSN motif, since a mutation of the serine into a large residue has been found to increase channel activity drastically (Kellenberger et al., 1999; Snyder et al., 1999; Hanukoglu and Hanukoglu, 2016).

Furthermore, the sodium-selectivity filter is located at an G/S–X–S- three residue motif, ensuring that only sodium but not potassium can pass the channel (Kellenberger et al., 1999). Moreover, the amiloride binding site is located within the TM2 domain. It is consistent of a serine, provided by the α- subunit and a glycine from both, β- and γ-subunit.

The TM2 domain, as well as the TM1 domain, contributes to ENaC's anchoring into the double layered lipid membrane by interacting with the hydrophilic head groups through three positively charged amino acids, located at the cytoplasmic side according to the 'positive inside' rule (von Heijne, 1992). This region also binds to phosphatidylinositol triphosphate, which acts as an allosteric activator for ENaC (Di Paolo and De Camilli, 2006).

Carboxy terminus

The carboxy terminus is mainly involved into protein-protein interactions, such as phosphorylation and ubiquitination, hence internalization and degradation of

ENaC, and by that is a major factor contributing to the half life time of the channel (Hanukoglu and Hanukoglu, 2016a).

It is phosphorylated by a lot of enzymes such as the serum and glucocorticoid-regulated kinase 1 (SGK-1), Phosphokinase C (PKC), extracellular signal-regulated kinases (ERK) and casein kinase 2 (Diakov and Korbmacher, 2004; Yang et al., 2006). The C-terminus of the β-subunit might be essential for the Cl-dependent inhibition of the channel (Bachhuber *et al.*, 2005).

Moreover, the C-terminus is thought to interact with the cytoplasmic proteins and it contains the proline-tyrosine rich motif 65 to 70 bases downstream the TM2 domain of all subunits (Figure 3). The recognition of the PY motif by Nedd4-2's WW-motif as well as the ubiquitination of the PY motif are considered to be essential for the internalization and the degradation of ENaC (Rotin and Staub, 2012; Hanukoglu and Hanukoglu, 2016b). The four investigated α-stop-codon mutants lack those important residues as well as the suggested critical binding sites of TIP-peptides (Lucas et al., 2016), but still AP318 shows significant effect in electrophysiological experiments.

Regulation of ENaC - Overview

The epithelial sodium channel ENaC is known to be regulated on many levels. To break it down, two different mechanisms are distinguished. First, there is the slow genomic modulation of ENaC which shows up after hours to days after induction and secondly there is an early, nongenomic activation of ENaC which can be seen in less than 10 minutes after induction (Hamm et al., 2010). The comprehension of those mechanisms is mandatory to understand the symptoms PHA1B patients show.

The genomic modulation is characterized by an upregulated gene expression of regulatory proteins and includes translational and posttranslational modifications in response to the body's biophysical state, while as nongenomic regulation we understand influencing the protein assembling, cell trafficking, cleavage, ubiquitination, degradation, activation and recycling of ENaC (Hamm et al., 2010).

Aldosterone, serving as a good specimen, is very well known to be the main hormone in ENaC regulation and as such to boost the genomic machinery of ENaC, thus raises the intracellular protein expression and overall number of matured channels integrated into the membrane. Though, it also has acute activating properties towards sodium currents (Verrey *et al.*, 2008).

The non-genomic regulation mainly influences cell trafficking due to a variety of different mechanisms, as well as activation by proteases or due to mechanosensitivity, hence is very complex and not less important than the genomic one.

Aldosterone

Aldosterone is generally accepted to be the most important hormone to influence ENaC. As a mineralocorticoid hormone it is lipophilic enough to pass the cells membrane and to directly interact with a cytosolic mineralocorticoid receptor (MR), which translocates into the nucleus (Galigniana *et al.*, 2004). There it binds to the mineralocorticoid responsive element (MRE) on the DNA, serving as a promotor to increase the synthesis of MRE dependent mRNA, initiating the expression of a variety of proteins.

Intracellular ENaC regulating pathways

This section discusses mechanisms and cascades which control ENaC's expression and activity.

Protein Kinase A

Due to Aldosterone, the intracellular cyclic adenosine monophosphate (cAMP) rises, which is necessary for the activation of protein kinase A (PKA) (Figure 6).

Protein kinase A is activating a bunch of target proteins by phosphorylating them. As a consequence, ENaC is not only synthesized in a larger number, but also it is translocated from a subapical pool to the membrane, increasing sodium

reabsorption (Debonneville *et al.*, 2001; Lu *et al.*, 2007; Hanukoglu and Hanukoglu, 2016).

SGK1/Nedd4-2 pathway

The SGK1-Nedd4-2 pathway plays an important role in the recycling of ENaC. The pathway is responsible for both, degradation of membranal ENaC, as well as a quick replenishment of channels into the membrane (Lu *et al.*, 2007b).

Neural precursor cell expressed developmentally down-regulated protein 4 (Nedd-4-2) is an ubiquitinylating enzyme that provides ENaC at its PY motif with an ubiquitin rest. Ubiquitinylated proteins migrate to the lysosomes and/or proteasomes, where they get degradated. Hence, Nedd-4-2 decreases ENaC cell surface presence and ENaC dependent sodium reabsorption. It has been shown that ENaC does not run through a real recycling, but degradated channels will never reappear (Lu *et al.*, 2007). More likely, there is a subapical storage of fully glycosylated, matured ENaC from which channels can be rapidly inserted into the membrane (acute effect of aldosterone through SGK-1). This subapical pool can be refilled by aldosterone due to enhanced protein synthesis (chronic effect of aldosterone). If the PY motif is mutated, ENaC cannot be degradated (since there is no Nedd-4-2 dependent ubiquitination), and therefore the mutated ENaC stays in the membrane up to four times longer than WT ENaC, causing a high sodium absorption, thus high blood pressure, known as Liddle syndrome (Lu *et al.*, 2007).

The PY motif also seems to play a major role in the acute effect of aldosterone, thus replenishment of channels. It has been shown that in Liddle syndrome associated ENaC mutants, at which the PY motif is mutated, the recruitment from the subapical pool is barely taking place. The high ENaC activity, which induces high blood pressure, is only caused due to not internalising functional ENaC (Lu *et al.*, 2007).

The serum and glucocorticoid-regulated kinase 1 (SGK-1) (Hanukoglu and Hanukoglu, 2016; Wynne *et al.*, 2017) has several mechanisms with which it positively regulates ENaC's expression. SGK-1 dependent phosphorylation of Nedd 4-2 inhibits its induced degradation of the protein, hence increases the

overall count of functional channels in the membrane (Figure 6; Figure 7) (Lang *et al.*, 2006) as well as the sodium reabsorption. Due to the induction by aldosterone, SGK-1 rapidly recruits the subapical stored ENaC channels into the membrane (if the channels possess the PY motif) (Lu *et al.*, 2007; Valinsky et al., 2018). Due to that, SGK1 is responsible for aldosterones acute effect on ENaC. In the long run, via SGK1, the transcription of Dot1a/AF9 is downregulated, increasing the synthesis of α-ENaC subunits.

SGK-1 phosphorylates WNK4, attenuating the inhibitory function of WNK4 on ENaC (Valinsky et al., 2018).

It was also shown, that SGK-1 might directly activate the α-ENaC subunit, thus enhances currents up to a 2 fold, without affecting open channel probability by converting silenced channels into active ones (Valinsky et al., 2018). This once again calls for a fast replenishment of channels.

Dot1a / AF9 inhibitory transcription complex

Dot1a is a recently described aldosterone dependent histone modifying protein. *In vivo* and *in vitro* it is colocalized with AF9, a putative transcription factor, in renal cells (mIMCD3). Dot1a is an early repressor of α-subunit transcription due to a histone H3 Lys-79 associated hypermethylation at α-ENaC's promotor, and thus decreases the level of α-ENaC mRNA expression (Zhang et *al.*, 2006; Hamm et al., 2010; Shibata and Fujita, 2011b; Valinsky et al., 2018). AF 9 knockdown by interference mRNA shows opposite effects indicating a synergetic effect of Dot1a and AF9 (Zhang *et al.*, 2006). Aldosterone via SGK-1 decreases Dot1a / AF 9 expression, hence leads to Dot1a / AF 9 histone H3 Lys-79 targeted hypomethylation and due to that enables α-ENaC- subunit transcription (Zhang *et al.*, 2006).

WNK-1 / WNK-4 molecular switches

With no lysine Kinase (WNK) is a kinase family which was found by accident just a couple of years ago (Hoorn *et al.*, 2011).

Nowadays WNK's major role in regulating ENaC activity as well as other protein channels located in the kidneys epithelial membrane such as Sodium-Chloride-Cotransporter (NCC), Sodium-Potassium-Chloride Cotransporter (NKCC) and renal outer medullary potassium channel (ROMK), is well known (Hoorn *et al.*, 2011). WNK is most abundant in the amiloride sensitive distal convoluted nephron. As kinases the WNK 1-4 family phosphorylates target proteins such as STE20/SPS1 and SPAK (STE20/SPS1-related proline/alanine-rich kinase)/ OSR 1 (oxidative stress responsive protein 1), hence regulates their activity. As a main influence on ENaC, WNK1 stimulates SGK1 (Figure 7) via the PI3K (Phosphoinositol 3 Kinase), therefore upregulates ENaC while WNK 4 inhibits ENaC (which is reversed in the presence of SGK1) (Hoorn et al., 2009; Hoorn and Ellison, 2012).

GILZ 1

Aldosterone also increases the synthesis of GILZ1, a member of the TSC22 family of leucine zipper proteins.GILZ1 inhibits the Raf dependent activation of MEK and thereby Erk 1 / 2 effect on ENaC (Soundararajan et al., 2011). Erk seemingly phosphorylates the channel, hence favours the interaction between Nedd 4-2 and ENaC due to what ENaC gets degradated (Figure 6). The GILZ 1 dependent action results in an increased ENaC activity (Hoorn and Ellison, 2012).

USP 2- 45

Another protein that has been identified to increase ENaC's activity due to aldosterone dependent induction is the USP 2- 45. It has been found in a gene expression screen in mice and it was shown, that it also has its effect in *Xenopus Laevis* oocytes when coexpressing ENaC. USP 2-45 seems to hinder Nedd-4-2 dependent channel degradation (Figure 6) the same way SGK1 does, since it has not increased ENaC activity when devoid of the PY motif containing C-terminus nor N- terminus, which also includes possible ubiquitinylation sites (Verrey *et al.*, 2008).

K-Ras pathway

The small G-protein Ras plays a major role in a lot of pathways including cell proliferation, activation of target proteins, differentiation apoptosis and carcinogenesis. One of more than a hundred of related proteins that have been found over time is the K- Ras (Gormley et al., 2003). As typical for that protein family, K-Ras is activated when binding GTP. As a result, active K-Ras induces 2 regulatory pathways: it activates PI3K / PDK1/2, hence raising SGK1 and ENaC activity. Also K-Ras stimulates the ENaC Raf – MEK- Erk 1/2 dependent phosphorylation of ENaC, hence degradation by Nedd-4-2 ubiquitination (Figure 6) (Verrey *et al.*, 2008).

14-3-3

SGK-1 mediated phosphorylation of Nedd4-2 initiates 14-3-3 binding and thereby inhibits Nedd4-2 interaction with ENaC, which results in an indirectly increased ENaC activity (Figure 6) (Shibata and Fujita, 2011).

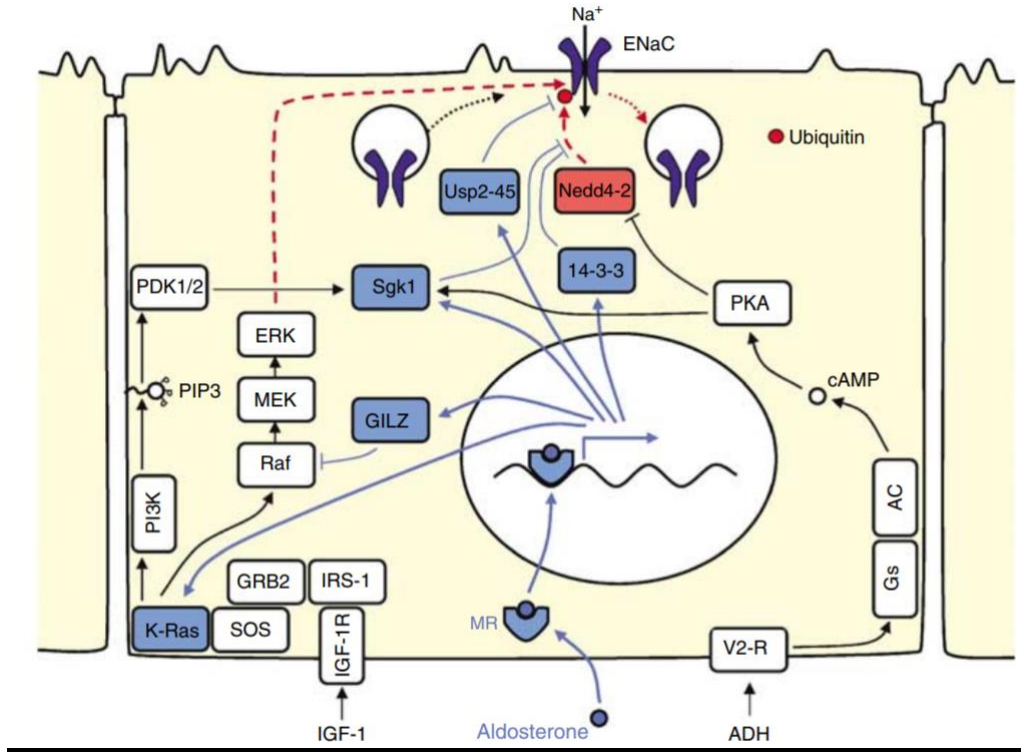

Figure 6 ENaC regulatory mechanisms (Shibata and Fujita, 2011)

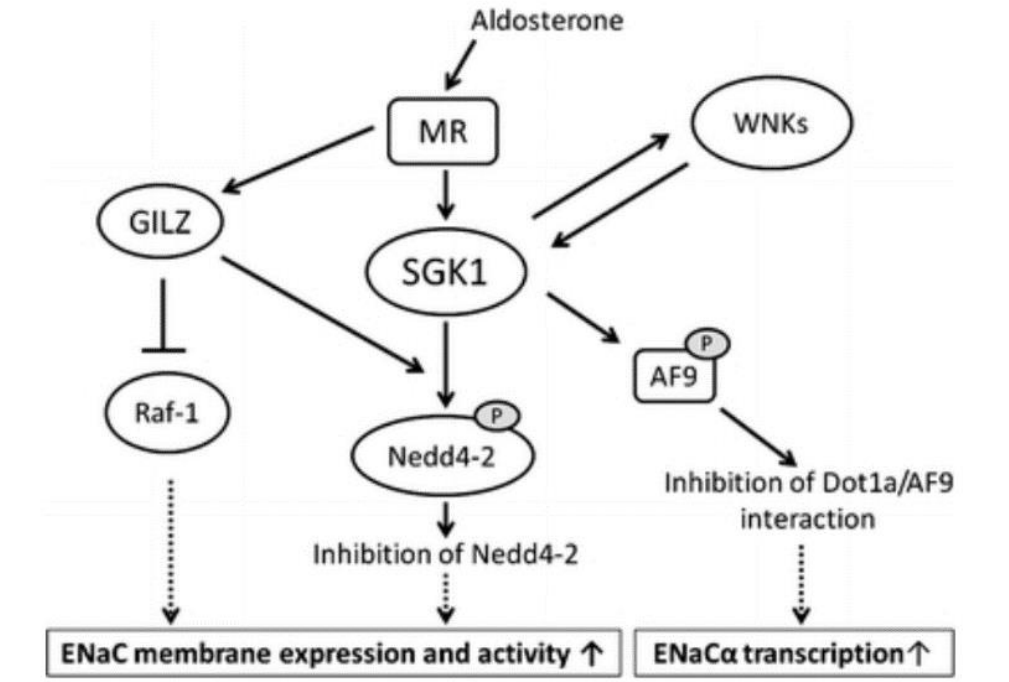

Figure 7 ENaC-expression regulatory pathways (Verrey *et al.*, 2008)

Phosphoinositides – PIP2

Phosphoinositides exert a mandatory function on ENaC activity by affecting open probability and decrease retrieval of the channel. ENaC is sensitive to both, phosphatidylinositol 4,5-bisphosphate (PIP2) and Phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP2 directly interacts with the channel, enhancing open probability. The genetic deletion of another target of PIP2 - the P2Y2 receptor – is related to hypertensive conditions, since more PIP2 might be available to active ENaC, hence induces sodium reabsorption (Pochynyuk et al., 2008). PIP3 on the other hand does both, increasing number of the channels as well as activates it through a distinguished binding site than the one from PIP2. PIP3 represses channel retrieval by activating PDK1 and as a consequence SGK-1, which as described above increases cell surface presence of ENaC. PIP3 also rapidly activates ENaC in patch clamp experiments (Ma et al., 2002)

Mechanosensitivity

It has been shown, that ENaC is mechanosensitive, like some other proteins from the degerin superfamily. This is thought to be an important non-hormonal regulating mechanism, besides channel cleavage. Since the study of Fronius and Clauss in 2008 it is still controversial whether ENaC activation is induced by a membrane stretch caused by hydrostatic pressure, cell swelling, or due to shear stress (Fronius and Clauss, 2008). However, it was shown that fluid flow imitating physiological conditions increases ENaC open probability in perfused rat collecting ducts as well as in *Xenopus Laevis* oocytes expressing ENaC (Althaus *et al.*, 2007). Mechanosensitivity is a major non-genomic regulatory mechanism of ENaC.

Aldosterone paradox

The fact that the WNK network acts as a molecular switch on multiple ion channels like ENaC, NCC, ROMK, NKCC amongst others (Hoorn *et al.*, 2011) might play a big role in ENaC and nephron science in the future. It was figured out, that depending on the secretory stimulus of aldosterone, the WNK1 / Ks-WNK1 ratio either increases or decreases, causing the so called aldosterone paradox (Figure 8). In both pathophysiological states, hypovolemia and hyperkalemia, the aldosterone level is elevated, which induces different effects. In the appearance of AT II during hypovolemia, aldosterones major effect is sodium retentive by stimulating ENaC (and NHE3). In contrast, during hyperkalemia, aldosterone primarily acts potassium depletive while the WNK1 / Ks-WNK1- ratio decreases (due to a high potassium level) (Mccormick et al., 2008; Hoorn *et al.*, 2011). PHA1B patients show both symptoms, but critical aldosterone unresponsiveness.

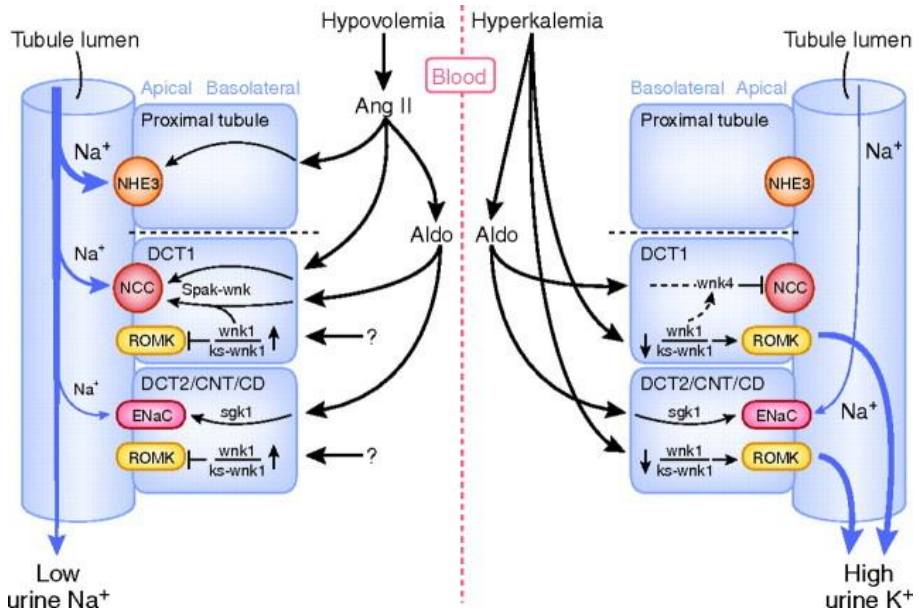

Figure 8 Aldosterone Paradox (Hoorn et al., 2011)

TNF-α

Tumor necrosis factor-α (TNF-α) belongs to the cytokine family and is a major acute phase cell signalling protein, derived mainly by macrophages and other immune cells, which is involved into processes such as cell proliferation, cell differentiation and apoptosis. In many known chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis a TNF-α dependent inflammatory cytokine/protein cascade is upregulated and due to that TNF-α antibodies are an important therapeutic field.

Besides the wide variety of effects TNF-α has within the human body, it is suggested that the interaction between TNF-α and ENaC's α-subunit is mandatory for its effect on the channel's membrane abundance. In the αN511Q mutant and α-ENaC lacking all five glycosylation sites, TNF-α effect on the ENaC membrane presence was vanished (Willam *et al.*, 2017b). TNF-α was shown to activate the channel with very high potency (6,7nM) (Willam *et al.*, 2017b). Though, TNF-α had a lower EC_{50} on sodium currents than Solnatide, the maximal induced current was higher with AP301 treatment (Willam *et al.*, 2017b).

Solnatide (AP301) and AP318

Solnatide and its congener AP318, a chemical derivate of Solnatide, are compounds developed by the company Apeptico. They are classed among the so called TIP- Peptides (TNF-α lectin like domain derived proteins).

TIP- Peptides are synthesized, cyclic proteins mimicking the lectin like domain of TNF-α (Figure 9). AP301 and AP318 activate ENaC by binding to the channel and stabilize its open probability / open conformation. They were shown in previous studies to rescue a PHA1B related loss of function ENaC (Willam *et al.*, 2017a).

It has been shown, that the first as well as the 17th amino acid of TIP peptides are important for binding affinity aspects, hence activating the channel. A free positively charged N-terminal amino group on residue one and/or a free negatively charged carboxyl group at residue 17 are essential to stimulate the channel (Hazemi *et al.*, 2010).

On ENaC's part there are two important characteristics mediating a channel activation by TIP- peptides:

1) Five N-glycosylated residues reaching from the extracellular loop to the carboxy terminus of the α-subunit, αN232, 293, 312, 397, 511- most importantly Asp_{511}- are necessary for the interaction between TIP-Peptides and the channel (Shabbir *et al.*, 2015; Willam *et al.*, 2017b).

2) three crucial residues between the pre-TM2 segment of ENaC, including Val_{567}, Glu_{568}, and Glu_{571} and the intracellular carboxy terminus of α-ENaC (Lucas et al*.*, 2016).

The original TIP peptide designed by Apeptico and synthesized via solid phase synthesis, was Solnatide (AP301), CAS Registry Number: 259206-53-6. It is derived from the residue C101 to E116 from the original human TNF-α (CGQRETPEGAEAKPWYC, AP301; CQRETPEGAEAKPWYE, hTNF-α). Solnatide is being tested in clinical phase IIb as a treatment of acute lung injury, acute respiratory distress syndrome and pulmonary oedema caused by lung infection or lung transplantation. It contains a disulphide bond, formed by cys1 and

cys 17, which makes the compound chemically instable to oxidations, hence is not optimal for a therapeutic to be used differently than by inhalation.

For that reason, chemical derivates of Solnatide, such as AP318 (Cyclo (4-aminobutanoic acid-GQRETPEGAEAKPWYD), were developed. In contrast to AP301, AP318 is lacking a disulphide bond from residue one to seventeen, which makes it chemically more stable to oxidation reactions. Previous won data from the Department of Pharmacology and Toxicology of University of Vienna show a lower EC_{50} of AP318 than AP301, thus indicating a stronger effect of AP318 on ENaC dependent sodium currents than of AP301. They also differentiate in a time dependent manner, since AP301 showed an effect within 10 minutes while AP318's effect appeared later but was stronger (Hazemi *et al.*, 2010).

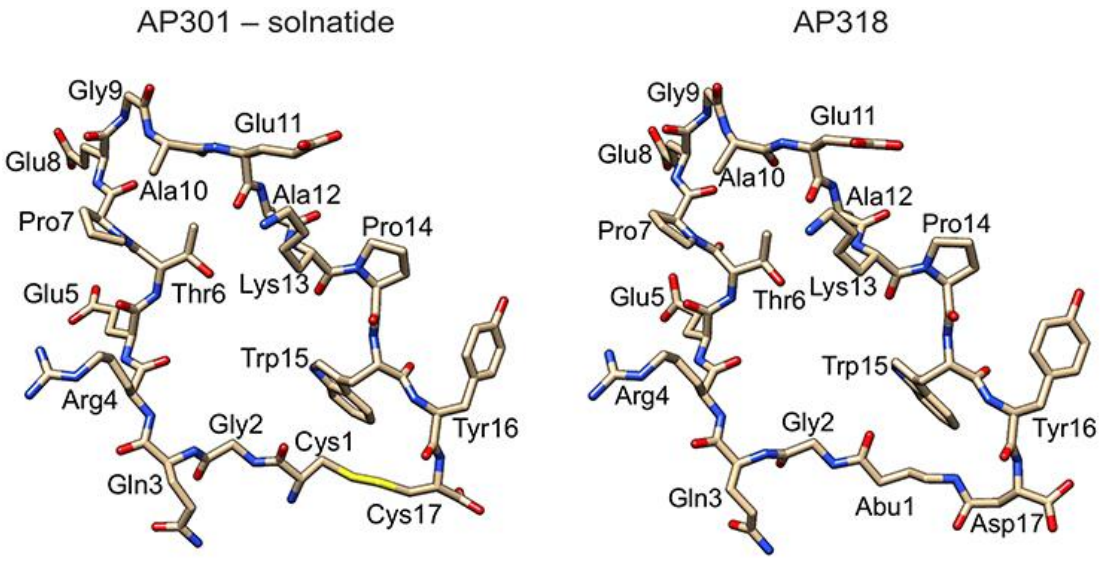

Figure 9 Structure of AP301 and AP318 (Hazemi *et al.*, 2010)

Material

In this part of the scientific work materials that were used are listed. They were ordered from Sigma Aldrich®, Bio-Rad®, AGFA®, Gibco® - Thermo Fisher®, Santa Cruz Biotechnology, Inc®

- Separating gel 12,5%:

Aqua bidest.	4,2ml
30% Acrylamide	3,3ml
Lower Tris buffer	2,5ml
10% SDS (sodium dodecyl sulfate)	100µl
TEMED	10µl
10% APS (ammonium persulfate)	100µl

- Stacking Gel, 4,0%:

Aqua bidest.	6,1ml
30% Acrylamide	1,3ml
Upper Tris buffer	2,5ml
10% SDS	100µl
TEMED	10µl
10% APS	100µl

- Upper Tris buffer (pH=6,8)

Tris-base (Sigma Aldrich®)	60,5g
10% SDS	4,0g
dH_2O	Add up to 1000ml

- Lower Tris buffer (pH=8,8)

Tris-base (Sigma Aldrich®)	36,4g
dH_2O	100ml
37% concentrated HCl	Adjust pH to 8,8
SDS	Add 0,8g SDS

- Secondary antibody

BSA 3% (bovine serum albumin)	2ml
TBST (Tris-buffered saline with Tween 20)	8ml
antibody for 1:10000 dilution	1µl

- 3% Blocking Solution

BSA	1,5g
TBST	50ml
NaN_3 (0.02%)	100µl

- 1M Tris-Cl (pH=7,5)

Tris-base (Sigma Aldrich®)	121,1g
dH_2O	800ml
37% conc. HCl	Adjust pH to 7,5

- 10x TBS

NaCl	87,7g
1M Tris-Cl (pH=7,5)	100ml
dH_2O	Add up to 1000ml

- 1x TBS

10x TBS	100ml
dH_2O	Add up to 1000ml

- 1x TBST

TBS	100ml
dH_2O	Add up to 1000ml
Tween® 80	1ml

- 10% SDS

SDS	1g
dH_2O	10ml

- 10% APS

APS	100mg
dH_2O	Dilute in 1ml

- 10% NaN_3

Sodium azide	100mg
dH_2O	Dilute in 1ml

- 10x Running buffer

Tris-base	60,0g
Glycine	288,0g
SDS	20,0g
dH_2O	Add up to 2000ml

- 1x Running Buffer

10x Running buffer	100ml
dH_2O	Add up to 1000ml dH_2O

- Towbin Buffer

Tris-Base	3,0g
Glycine	14,1g
SDS	1,0g
Methanol	200ml
dH_2O	800ml

- 0.2M Sodium Acetate (pH=5,0)

Sodium acetate trihydrate	1,4g
Glacial acetic acid	0,3ml
dH_2O	add up to 50ml

- 4x Sample Buffer

Glycerine	4,0ml
Upper Tris Buffer	4,8ml
SDS	0,8g
Bromophenol blue	4,0mg
β-Mercaptoethanol (laminar)	0,5ml
dH_2O	0,7ml

- Developer (protected from light)

DEV(AGFA®)	25ml
dH_2O	125ml

- Fixer (protected from light)

FIX(AGFA®)	30ml
dH_2O	150ml

- Biotinylation Kit (Sigma Aldrich®)
- 10% Triton X-100 (Sigma Aldrich®)
- QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies®) (#210519)
- Restriction enzymes: Bam HI, EcoRI provided by Dr. Annette Hohaus
- AP318 (Apeptico®)
- Anti-α-ENaC Antibody (Sigma Aldrich®)
- ECL solutions from Bio-Rad®
- DNA vector: pcDNA 3.1His/Max (Thermo Fisher®)
- αβγ-WT ENaC DNA kindly gifted from Peter Snyder, Iowa City VA Medical Center
- α-stop-codon mutants αR508X, αR492X, αV248X and αC63X provided by Anita Willam
- Primer for site directed mutagenesis (Agilent Technologies®)
- Sequencing at LGC genomics®, Berlin, Germany
- Anza™ T4 DNA Ligase Master Mix (Thermo Fisher®)
- QIAamp DNA Mini Kit from (QUIAGEN®)
- Nanodrop instrument (ThermoScientific®)
- ChemiDoc™ Touch Imaging System (Bio-Rad®)
- ImageJ developed by Wayne Rasband

Methods

Cell culture

The entire experiments were performed with HEK-293 cells (passage 7 to 38), as they are an established cell line for ectopic and heterologous protein expression due to their easy growing and transfection properties. HEK cells are human embryonic kidney cells, in which DNA from Adenovirus 5 (4.5 kilo bases into humans chromosome 19) was brought in.

CHO (Chinese hamster ovaries) cells were used for two experiments, but since they did not show any advantage over HEK cells, such as less unspecific bands in cell lysate experiments, only HEK cells were used in further experiments.

1) HEK cells and CHO cells were defrosted from -196 °C (liquid nitrogen) on ice. When only the core was still solid, cells were transferred into a cell culture flask containing growth medium (DMEM), supplemented with 10% FBS, 1% Penicilline/Streptomycine.

2) Cells were incubated at 37°C, 5% CO_2 in DMEM (Dulbecco's Modified Eagle Medium) + 10% FBS (fetal bovine serum) + 1% Penicilline/Streptomycine.

3) Every other day the confluency of cells in the T25 or T75 flasks was observed under the light microscope. Whenever it was higher than 90 percent, cells were splitted into a new flask by first sucking off the medium under the laminar air flow. Next, cells were washed with 2ml (T25) phosphate buffer saline (PBS). After that, cells were detached from the flasks by adding 0.5ml trypsin onto the top of them, followed by an incubation time of 5 minutes at 37°C, 5% CO_2 within the incubator. The detachment was controlled under light microscope. The cells were resuspended in 4.5ml fresh DMEM with a serological pipette. 1ml of the cell suspension was transferred into a new flask containing 4ml of DMEM. New cell suspension was put into the incubator at 37°C, 5% CO_2.

Cell seeding for transfection

HEK cells were splitted as previously described into 100mm cell culture plates (0.5ml cell suspension plus 8ml DMEM within each plate) and put back into the incubator at 37°C, 5% CO_2.

Transfection

24 to 48 hours post seeding, when the confluency was between 75 and 90 percent, cells were transfected with either his-tagged-α-WT or PHA1B α-stop-codon mutations in combination with βγENaC WT DNA.

240µl serum free medium was pipetted into a 1.5ml microcentrifuge tube for each 100mm cell culture plate for a transient transfection. 3µg of the α-subunit and 700ng of βWT and γWT was added, to guarantee an average channel stoichiometry of αβγ-ENaC. 6µl of Roche® x-treme Gene transfection reagent HP was added to the DNA and incubated for 30’ at room temperature.

Under the laminar airflow, the medium was sucked off from the cell culture plates and the cells were washed with 4ml PBS twice.

The transfection complex was gently added onto the cells one drop at a time to prevent detachment of the cells from the plates and guarantee an ideal protein expression after 48 hours.

One day post transfection (when the confluency was above 60%), the cells were splitted, to achieve similar transfection- and expression efficiency of all WT samples and all mutant samples.

Treatment

48 hours post transfection the cells were treated with the compound AP318 for either 5, 10 or 30 minutes. Subsequently cell surface proteins were biotinylated using Sigma-Aldrich‘s biotinylation kit in order to perform membrane protein isolation. That was done to prevent unspecific protein bands in western blot

experiments and to only measure ENaC channels which are embedded into the membrane.

The medium was sucked off from the plates and the cells were washed with 4ml PBS twice. AP318 was diluted in DMEM (+10%FBS +1%Pen/Strep) to a total concentration of 200nM. This was done by diluting 6µl AP318 from a 1mM stock solution in 30ml DMEM. 10ml of the resulting solution was put onto the HEK-293 cells for 5, 10 and 30 minutes. In the first experiment the optimal application time for AP318 was determined, whereas AP318 showed a transient effect (expression decreased within the period of 30 minutes to 60 minutes of treatment), which is why in the following experiments 30 minutes was chosen as the maximal treatment time. After the respective time controls (either 5, 10 or 30 minutes of treatment), the medium, containing AP318, was sucked off from the plates and cells were washed once with PBS.

Protein harvesting - Cell lysate

The confluency of the cell culture was controlled under the light microscope. When it was above 70 percent, medium was sucked off under the laminar air flow and cells were washed twice with 4ml PBS (phosphate buffer saline). All further steps were performed in a non-sterile environment. Depending on the confluency of the cells (75-100%), 400 to 600µl sodium acetate was dropped onto them. Cells were incubated on ice for 2 minutes. The suspension was translocated into a 1.5 microcentrifuge tube. Protease inhibitor cocktail from Roche® was added in a 1:10 ratio. The cells were exposed to three 10-seconds-lasting-sonication bursts with 30 seconds incubation time on ice in between. Cells were vortexed and Triton X-100 was added in a 1:100 ratio to Na-acetate. Cells were incubated on ice for 30 minutes, then centrifuged at 4°C, 12500 RPM for 30 minutes. The supernatant was transferred to another 1.5ml microcentrifuge tube and stored at -20°C.

Protein harvesting – Biotinylation

Biotinylation was performed with EZ-Link™ Sulfo-NHS-SS-Biotinylation Kit from Thermo Fisher®. One vial of Biotin was dissolved in 50ml ice cold PBS. Cells were washed twice with 4 ml PBS. Onto each plate 2.5ml Biotin-Solution was added and the plates were incubated at 4°C for one to two hours. After dropping 125µl of quenching solution onto each plate, the cells were scraped into a 10ml tube. Dispersion was centrifuged for 3 mins at 500x g. 5 ml PBS was added to the cell pellet and gently pipetted up and down, centrifuged 3 mins at 500x g and the supernatant was discarded. 125µl lysis buffer containing 12.5µl (1:10) Protease Inhibitor Cocktail from Roche ® was added to the cells. Afterwards, the suspension was transferred into a 1.5ml microcentrifuge tube and pipetted up and down gently. Five 1-second-sonication bursts were performed at low power to disrupt the cells.

Then cells were incubated on ice for 30 minutes, while another 1-second-burst and vortexing was performed every 5 to 10 minutes. Cell lysate was centrifuged at 10000x g for 2 minutes at 4°C. Clarified supernatant was transferred into a new 1.5ml microcentrifuge tube containing the beads.

100µl Streptavidin beads were centrifuged in a 1.5ml microcentrifuge tube and washed with 1 ml PBS.

The proteins were mixed with the beads over night at 4°C on a rotating machine.

The next day, the proteins were centrifuged twice and the supernatant was discarded. The pellet was resuspended in a solution containing 1x sample buffer (250µl 4x SB + 750µl bidest. H_2O) + 100µl Inhibitor Cocktail from Roche ®. Proteins were centrifuged and denatured at 65°C for 10 minutes before loading them onto the electrophoresis gel.

Western Blot

Western Blot is a semi quantitative technique to detect specific proteins of a sample mixture. The harvested proteins are coated with a high amount of negative charges from sodium dodecyl sulfate and run in their denatured form on a polyacrylamide gel in gel electrophoresis, where they get separated based on their molecular

weight. Each protein gives a specific band, which can be detected with the corresponding antibody pair. The fingerprint of the resulting bands is transferred from the polyacrylamide gel onto a solid methylcellulose membrane. Finally, the proteins are marked on the membrane with primary and secondary antibodies to visualize them and for semi- quantification purpose. The primary antibody binds to a specific sequence of amino acids on the protein of interest, therefore only the proteins possessing those residues should become visible (Mahmood and Yang, 2012). The higher the amount of protein, the thicker and more intense the bands are, which can be used for semi quantification in order to compare protein expression of different samples – in our case the membranal ENaC expression of treated and untreated cells.

Therefore, a 12.5% running gel and a 4% stacking gel were prepared by mixing bidest. H_2O, upper/lower TRIS buffer, polyacrylamide, 10% SDS, 10% APS and TEMED in the recipe's ratio and letting them harden for one hour in Bio-Rad's plastic frame. Biotinylated proteins were denatured at 65°C for 10 minutes and put on ice. Gel was loaded with 20µl of protein samples and run with 200 Volt until the front reached the end of the glass plate.

Then, the gel was semi-dry blotted onto a methylcellulose membrane, which has been activated in Towbin buffer, by building a sandwich consistent of filter, membrane, gel and filter in a blotting cassette. The transfer was performed by setting 1 Ampere as consistent and going up from 8 to 25 V within 30 minutes. Negatively charged proteins are transferred from the cathode to the anode, hence from the gel onto the membrane. The employed marker was visible on the methylcellulose membrane after successful blotting.

Protein detection

After the transfer, the membrane was washed with TBST for 5 minutes to get rid of disturbing gel fragments. Afterwards the membrane's unspecific binding sites were blocked with 3% BSA and the membrane was incubated over night at 4°C.

The next day, the primary anti-α ENaC antibody (SigmaAldrich ®) 1:1000 was put onto the membrane and incubated over night at 4°C to specifically mark ENaC proteins.

The day after, primary antibody was poured back to reuse it. The membrane was washed three times with TBST for 5 minutes. Thereafter, secondary anti-rabbit – HRP- conjugated antibody was added onto the membrane (1:3000) by diluting 3.3µl antibody in a mixture of 2ml 3% BSA and 8 ml TBS.

Site directed mutagenesis: with traditional PCR

Site directed mutagenesis is a method often based on PCR which is used to insert a mutation into a DNA sequence (Bachman, 2013). This tool was used to get rid of the initial ATG at the beginning of the α-ENaC gene, which was ligated into pcDNA 3.1 vector, by using DNA binding primers, such which are about 30 amino acids long and already contain the mutation we wanted to insert.

Polymerase chain reaction – PCR

Polymerase chain reaction is a technique to amplify DNA or to insert specific mutations into a known DNA sequence using specific DNA binding primers (site directed mutagenesis). Those primers were theoretically created with the point forward method (and carried the mutation we wanted to insert) while Agilent Technologies® synthesized them.

PCR is divided into 3 steps which repeat over and over for about 18 cycles. Firstly, the denaturation (separating of the two DNA strands) is taking place at about 98°C. Secondly, the primer annealing happens after a cool down to 68°C. Forward and reversed primer attach to the DNA at this temperature. If a primer contains the mutation, it will subsequently be established into the genome during the extension phase by the DNA polymerase, which fills up the missing complementary bases to the template strand at 68 to 72°C. This process repeats repetitively, amplifying the region of interest exponentially.

Digestion (Restriction)

ENaC α-WT was cut out from the vector, which was kindly gifted from Peter Snyder, at its multiple cloning site by digesting it with the restriction enzymes BamHI and EcoRI. Afterwards, the product was ligated into the pcDNA 3.1 His/Max, which was also digested with the same enzymes. Then, using Anza™ T4 DNA Ligase Master Mix, both products were ligated together. 1µg of pc DNA 3.1 His/Max, 1µg of α-ENaC WT-vector, 2.5µl buffer, 1µl BamHI and 1µl EcoRI were pipetted into a 1.5ml microcentrifuge tube and added up to a total volume of 25µl with nuclease free water. Digestion was performed over night at 37°C.

Ligation

The digested DNA product was heat inactivated at 72°C for 5 minutes. The next day, a new 1.5ml microcentrifuge tube was prepared. 5µl of Anza T4 Ligase, 2.5µl digested DNA product and 12.5µl nuclease free water were added and ligated at room temperature for 30 minutes.

After ligation, site directed mutagenesis was performed, to get rid of the initial ATG of α-ENaC - such as above described.

Transformation

XL-10- Gold- Ultracompetent Cells (Agilent Technology) were taken from -80°C and put onto ice. 35µl cells were aliquoted into a 14 ml tube. 2µl Mercaptoethanol was added and incubated on ice for 2 minutes. 4µl ligated DNA product was added and incubated on ice for 30 minutes at room temperature. Afterwards, cells were exposed to heat shock for 30 seconds at 42°C. 500µl NZY^+ medium was added, cells were put into shaker at 37°C, 250rpm for 1 hour. Suspension was centrifuged, 400µl of the supernatant were discarded. The cell pellet was resuspended, put onto an ampicillin containing agar plate with sterile tools and put into the incubator at 37°C for 16 to 18 hours.

Colony picking

The next day (after 16 – 18 hours), if bacteria grew, a single colony was picked, using a pipette tip, and put into a glass tube containing 3ml of LB medium / 30µl of ampicillin. Tube was incubated at 37°C for 16-18 hours.

DNA extraction

DNA extraction was performed with QIAamp DNA Mini Kit (Qiagen) following the protocol. 2ml of the overnight culture was centrifuged for 2 minutes at 8000 x g, supernatant was discarded. To the cell pellet 250µl of lysis solution was added and mixed by inverting. 350µl neutralisation solution was added and mixed by inverting twice. Solution was centrifuged for 5 minutes at 13000 x g. Supernatant was transferred onto a column and centrifuged for 1 min (13000 x g), 500µl wash solution was added, column was centrifuged at 13000 x g for 1 min and flow through was discarded. Washing step was repeated. The empty column was centrifuged for 1 min (13000 x g). Dry column was put into a 1.5ml microcentrifuge tube. 20 µl elution buffer was added directly onto the membrane of the column. Column was incubated at room temperature for 2 minutes and afterwards centrifuged for 2 minutes (13000 x g). Column was discarded.

Nucleic acid quantification

DNA concentration was measured with a Nanodrop spectrophotometer (ThermoScientific®) loading 1 µl of sample and analyzing the fluorescent dependent absorbance.. DNA concentration was given as ng/µl.

Sequencing

The sequencing samples were prepared in a 2ml microcentrifuge tube mixing 1000ng of DNA with 7µl sequencing primer and added up to a total volume of 15µl with bidest. H_2O, if needed. Afterwards samples were labeled.

Samples were sent to LGC Genomics GmbH in Berlin, Germany and analyzed with standard Sanger sequencing. Results were provided after 3 days per email.

DNA Amplification

Same procedure as described above, including transformation of DNA into bacterial cells, colony picking, DNA extraction and measurement of extracted DNA with nanodrop measurement.

Statistical analysis

Three individual experiments were performed for each mutant. The blot intensities for ENaC expression and loading control (β-Actin) were evaluated with ImageJ. Significance was calculated with an unpaired t-test. The values are given in the results parts.

The significance of the following results was determined by:

- $p<0.05$ * significant
- $p<0.01$ ** very significant
- $p<0.001$ *** extremely significant

Results

Transfected HEK-293 cells expressing either wild type ENaC or PHA1B-α-stop-codon mutants in combination with βγ-WT were treated with AP318. Membrane proteins were isolated, separated on a SDS polyacrylamide gel, blotted onto a nitrocellulose membrane and detected with a pair of primary (rabbit anti α-ENaC antibody targeting the N-terminus of α-ENaC) and secondary antibody (anti-rabbit antibody, conjugated to horseradish peroxidase). Loading control: β-Actin.

Targeting α-ENaC with anti His/Xpress antibodies

When using anti-His antibody, ENaC WT and α-stop-codon mutant R508X were detected at a much smaller molecular weight (about 25kDa), than they were supposed to show up (75/90kDa for WT), (Figure 10). There was no difference in height and no difference between transfection reagents visible. Untransfected HEK cells showed a very marginal band in cell lysate at approximately same height (25kDa). Repetitive trials (n=3) showed the same results. All that indicates unspecific detection. Therefore, further experiments were performed with anti-α-ENaC antibody.

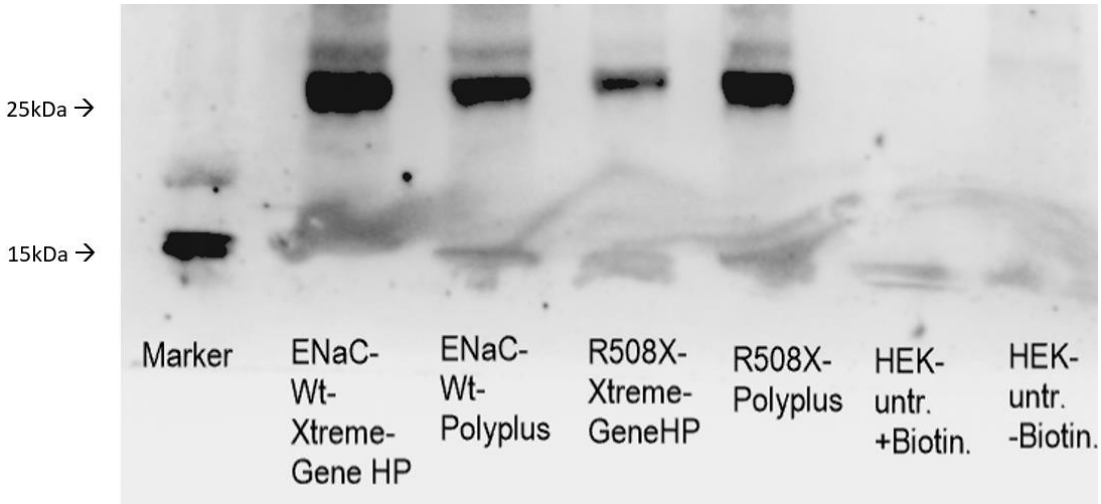

Figure 10 Detection of α-ENaC WT and αR508X with anti-His antibody

Effect of AP318 on membrane expression of ENaC mutant R508X

The blot visually shows an enhancement in membranal expression of WT (after 5, 10 and 30 minutes of treatment), but especially of the α-stop-codon mutant αR508X after 30 minutes of treatment with AP318 (Figure 11A). Due to the smaller molecular weight, the mutant migrated further than WT into the gel. Untransfected HEK cells do not give a band. Loading control was performed with β-Actin.

Membrane expression of ENaC WT and α-stop-codon mutant R508X before and after AP318 treatment was analyzed. There was a significant increase of WT expression (n=3; p=0,0035) after 30 minutes of treatment (Figure 11B). R508X membrane presence was significantly increased after 5 (n=3; p=0,0019) and 30 (n=3; p<0,001) minutes of treatment with the compound AP318 (Figure 11B). No significant difference in basal membranal expression of ENaC WT and mutant R508X was observed (Figure 11C). WT showed a significant difference in membrane abundance compared to αR508X, after both were treated for 30 minutes with AP318 (n=3; p=0,0077) (Figure 11C).

All shown bars in figures B) and C) follow the same scheme as indicated in figure A), respectively (Figure 11 A,B,C).

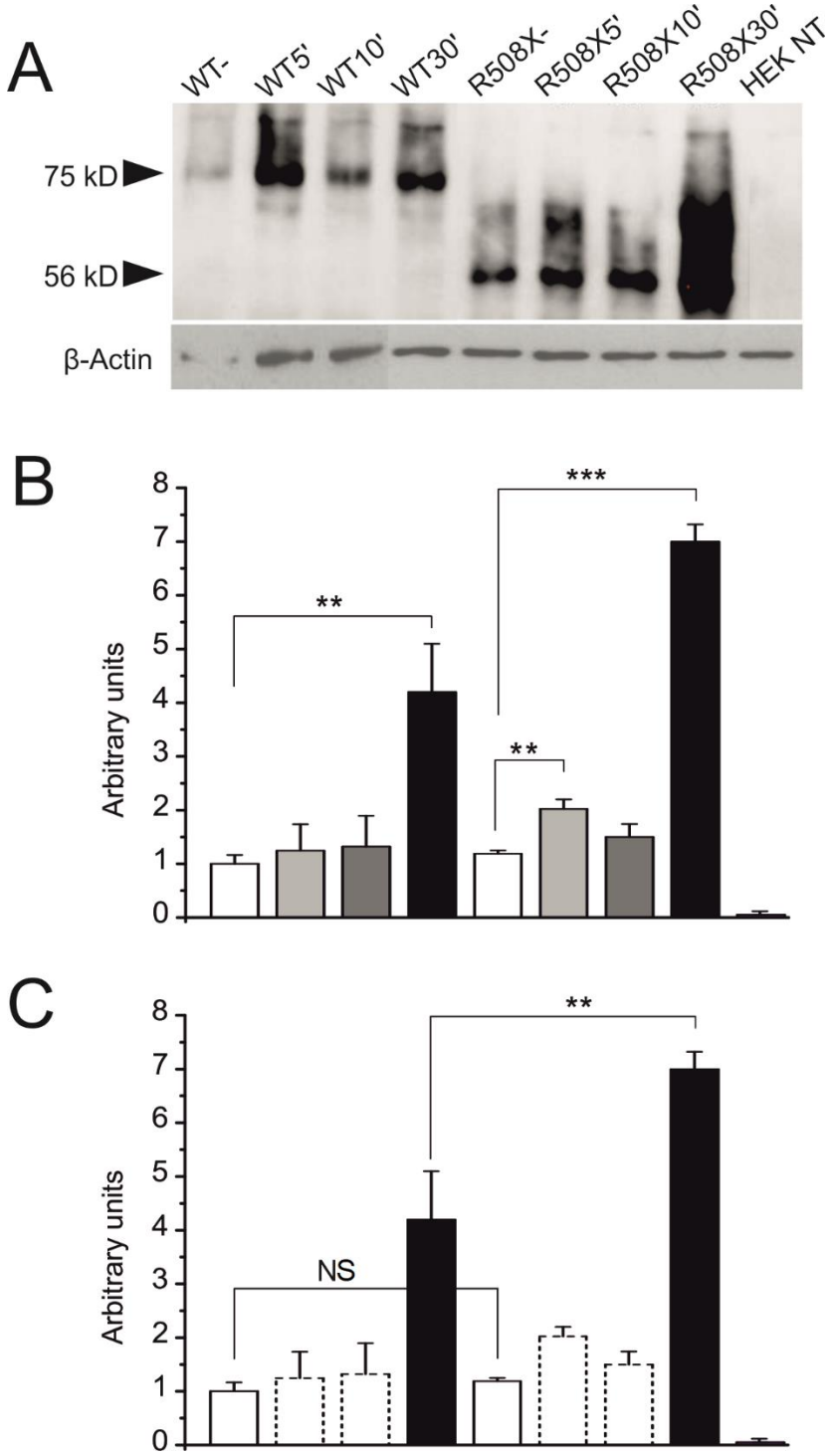

Figure 11 ***AP318 enhances membranal expression of ENaC WT and mutant αR508X. A)*** *A typical Western blot out of three with biotinylated surface proteins of HEK-293 cells transiently transfected with WT (-, control) or α508X mutant ENaC treated with AP318 at indicated time points is shown, using α-ENaC and β-Actin antibodies. NT; non-transfected* ***B)*** *Mean values ± SD of quantitation of 3 individual experiments of above shown western blots. AP318 (200 nM) induced expression of ENaC WT and αR508X was normalized relative to WT control (first empty bar). Membrane abundance was increased with 200 nM AP318 at indicated time points (5', 10' or 30'). Expression of αR508X ENaC was quantified relative to β-Actin. Significant differences are indicated using unpaired- t-test, *p < 0.05, **p < 0.01, ***p < 0.001, n=3.* ***C)*** *A comparison of in figure 11B quantified expression between WT and mutant (R508X) in both, control and treated for 30 min with AP318. For clarity the repeated bars are indicated with broken lines. NS; not significant.*

Effect of AP318 on membrane expression of ENaC mutant R492X

The membrane visually shows an enhancement in membranal expression of WT (after 5 and 30 minutes of AP318 treatment), but a more drastic one of α-stop-codon mutant R492X cell surface presence after 5, 10 and 30 minutes of treatment with AP318 (Figure 12A). R492X has a lower molecular weight than WT, which is why it migrated further into the gel. The bands detected at the start of the blot are most likely dimers at a molecular weight between 150 and 250 kDa. The bands indicated in the blot (75 and 56 kD) were used for quantification (Figure 12A).

There was a significant difference in basal membranal expression (n=3; p=0,0054) of ENaC WT and mutant αR492X as well as after both were treated for 30 minutes with AP318 (n=3; p=0,0026) detected (Figure 12C). Enhancement of αR492Xβγ was significant after all indicated time periods (n=3; p<0,001 / n=3; p<0,001 / n=3; p<0,001) (Figure 12B).

All shown bars in figures B) and C) follow the same scheme as indicated in figure A), respectively (Figure 12 A,B,C).

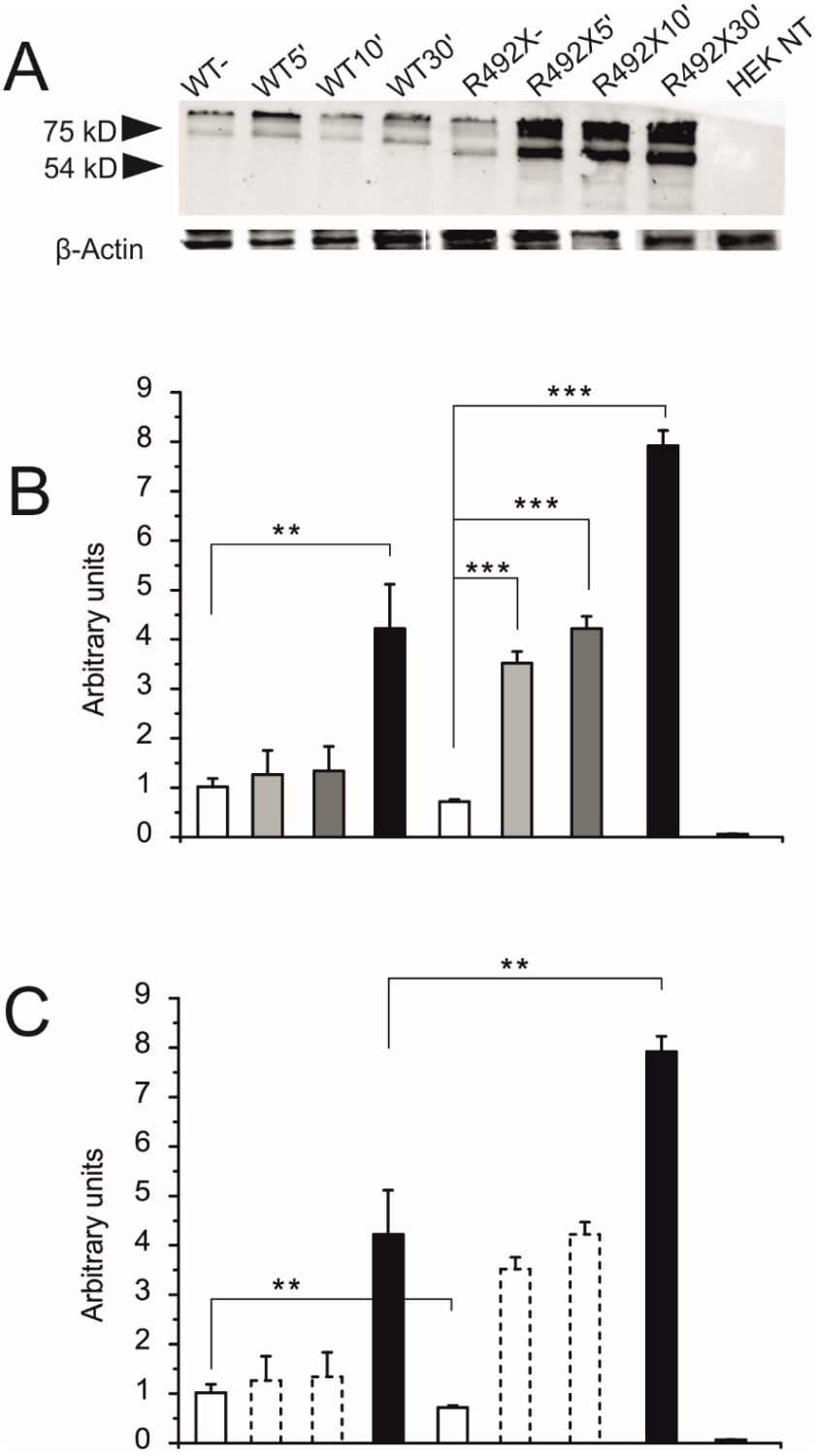

Figure 12 ***AP318 enhances membranal expression of ENaC WT and mutant αR492X. A)*** *A typical Western blot out of three with biotinylated surface proteins of HEK-293 cells transiently transfected with WT (-, control) or α492X mutant ENaC treated with AP318 at indicated time points is shown, using α-ENaC and β-Actin antibodies. NT; non-transfected* ***B)*** *Mean values ± SD of quantitation of 3 individual experiments of above shown western blots. AP318 (200 nM) induced expression of ENaC WT and αR492X was normalized relative to WT control (first empty bar). Membrane abundance was increased with 200 nM AP318 at indicated time points (5', 10' or 30'). Expression of αR492X ENaC was quantified relative to β-Actin. Significant differences are indicated using unpaired- t-test, *p < 0.05, **p < 0.01, ***p < 0.001, n=3.* ***C)*** *A comparison of in figure 12B quantified expression between WT and mutant (R492X) in both, control and treated for 30 min with AP318. For clarity the repeated bars are indicated with broken lines.*

Effect of AP318 on membrane expression of ENaC mutant V248X

The membrane visually shows an elevated expression of WT (75kD) after 5 and 30 minutes but a more drastic one in α-stop-codon mutant V248X (30kD) cell surface presence after 5 and especially 10 and 30 minutes treatment with AP318 (Figure 13A). Untransfected HEK cells do not give a band. V248X is much smaller than αENaC WT (669 amino acids), therefore the proteins migrated further into the gel. Another band, which is a little bit smaller than WT (visible as a single band at V248X control, 56kD), was detected which might be dimers of the mutant (494 amino acids → about the same weight as mutant αR492X; 56kD) (Figure 13A). Indicated bands at 75kD and 30 kDa were used for quantification.

A significant difference of V248X membrane abundance was observed after 5 minutes ($n=3$; $p<0,0001$), 10 minutes ($n=3$; $p=0,0001$), and after 30 minutes of AP318 treatment ($n=3$; $p=0,0001$), (Figure 13B).

Significant difference ($n=3$; $p<0,0001$) was detected in basal membranal expression of untreated WT and α-stop-codon mutant V248X as well as after both were treated for 30 minutes with AP318 ($n=3$; $p<0,001$), (Figure 13C).

All shown bars in figures B) and C) follow the same scheme as indicated in figure A), respectively (Figure 13 A,B,C).

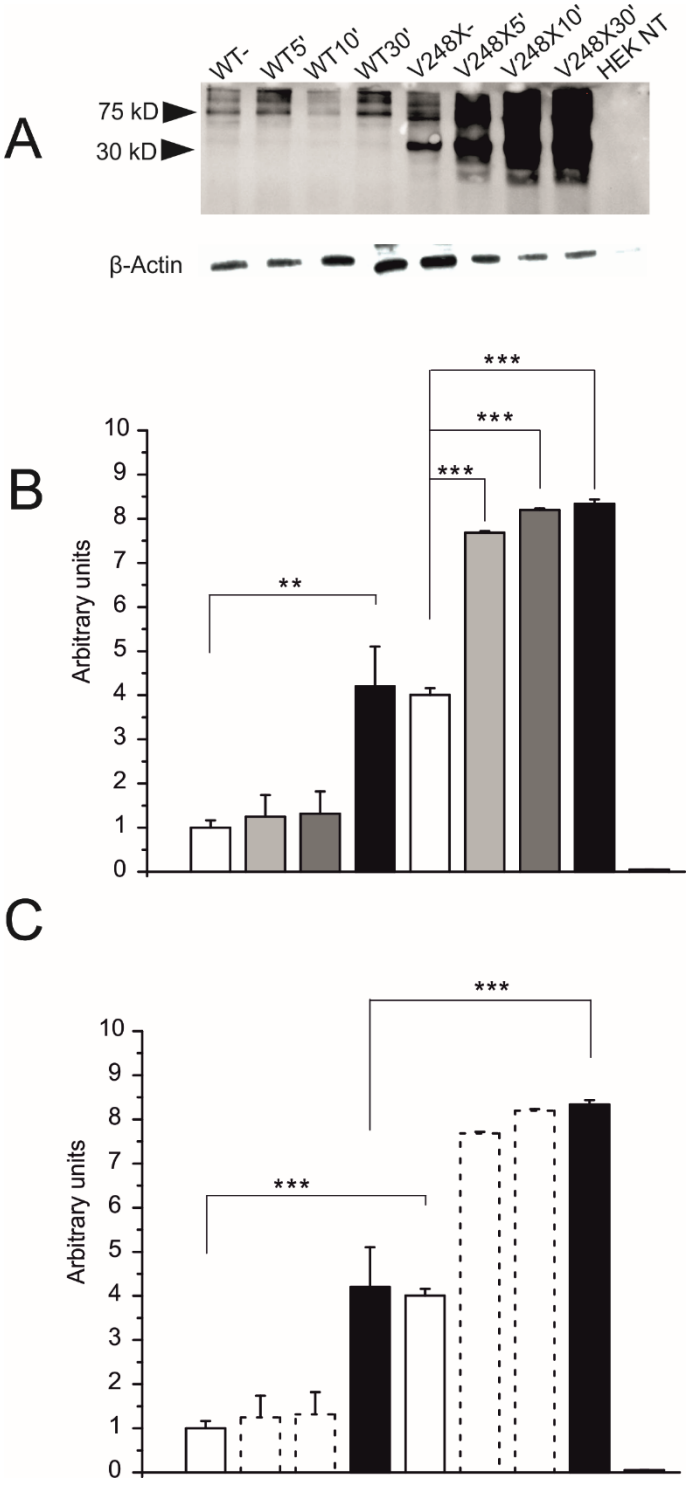

Figure 13 ***AP318 enhances membranal expression of ENaC WT and mutant αV248X. A)*** *A typical Western blot out of three with biotinylated surface proteins of HEK-293 cells transiently transfected with WT (-, control) or αV248X mutant ENaC treated with AP318 at indicated time points is shown, using α-ENaC and β-Actin antibodies. NT; non-transfected.* ***B)*** *Mean values ± SD of quantitation of 3 individual experiments of above shown western blots. AP318- (200 nM) induced expression of ENaC WT and αV248X was normalized relative to WT control (first empty bar). Membrane abundance was increased with 200 nM AP318 at indicated time points (5', 10' or 30'). Expression of αV248X ENaC was quantified relative to β-Actin. Significant differences are indicated using unpaired- t-test, *p < 0.05, **p < 0.01, ***p < 0.001, n=3.* ***C)*** *A comparison of in figure 13B quantified expression between WT and mutant (V248X) in both, control and treated for 30 min with AP318. For clarity the repeated bars are indicated with broken lines.*

Effect of AP318 on membrane expression of ENaC mutant C63X

Within four individual experiments, in which anti-α-ENaC antibody was employed, mutant αC63X could not be detected specifically. Both, untransfected HEK cells, which do not express ENaC WT, as well as αC63Xβγ show a band at the same height as visible for WT, indicating an unspecific detection of protein bands. No other band was detected for αC63X.

Discussion

The effect of the TIP-peptide AP318 on the membranal presence of ENaC WT and four α-stop-codon mutants in combination with βγ WT was investigated in this work. All acquired membranes coherently, visually and statistically show significant increase in surface expression of ENaC WT as well as three of the four investigated PHA1B related α-stop-codon mutants αR508X, αR492X, αV248X after treatment with a maximal effect after 30 minutes of treatment with AP318. In contrast, no specific band, thus no membranal expression of αC63X was detected in our western blot experiments, neither untreated nor treated one. In electrophysiological experiments αC63X dependent sodium currents also could not be derived. An explanation for that could be, that the cell does not detect this short protein (which is only about a 10th of the original subunit in length) as an endogenous ENaC protein and therefore ubiquitinylates and degradates it, without integrating it into the membrane. It is also possible that the proteins DNA does not get transcripted into mRNA or the protein is translated but not translocated and inserted into the membrane. The concentration of AP318 was 200nM in our experiments, which speaks for a good candidate as a future drug, since it provided high effects in our *in vitro* experiments at low concentration.

Previous studies suggest, that TIP peptides bind to α-ENaC subunit to stabilize its open conformation and enhance ENaC α-subunit expression, thus elevate sodium currents (Czikora *et al.*, 2014). That emphasizes the relevance of the investigated α-stop-codon mutants, since the V248X is lacking most of the N-glycosylation sites, as well as three important residues (Val_{567}, Glu_{568}, Glu_{571}) of the α-subunit, which are supposed to be mandatory for the interaction between TIP- peptides and the channel (Lucas *et al.*, 2016). Still, as visible in the shown blots and graphics, the membranal presence of the ENaC mutants increased by a fold due to AP318 treatment (Figures 11 – 13).

The information this thesis provides us with is a snapshot in time regarding membranal surface expression of ENaC before and after the treatment. It is not entirely clear, due to which effect the raised expression occurs - maybe an enhanced genetic expression of ENaC subunits is responsible for the strong elevation. Another possibility is an imitation of SGK-1 dependent integration of

subapically stored channels into the membrane, which increases membranal ENaC abundance. Though, a combination of both options is plausible.

During our work we had to confess, that recombinant α-ENaC was not specifically detectable with anti-His antibodies in our western blot experiments (Figure 10). Since we could detect His-tagged recombinant ENaC embedded into the membrane with an anti-α-ENaC antibody, most likely a problem of accessibility of the His-antibody towards the targeted epitope was present. It is possible, that the introduction of six positively charged histidine residues at the N-terminus induces a conformational change of the N-terminus, which is why the antibody cannot target the epitope anymore.

Hopefully AP318 shows the same positive effects regarding ENaC's activity and expression in animal models and subsequently in real patients, as it does *in vitro*. If the drug works as expected, it will be a big task to find a proper application form and strategy for it. Treating lung specific ENaC loss of function, allows inhalation of TIP peptides (AP301), due to which the drug reaches its target organ easily. However, in PHA1B patients the kidneys and the lungs, amongst other tissues, have to be addressed simultaneously. Since AP318 is a peptide drug it might be degradated by carboxy- and pancreatic peptidases, when applied orally. Injecting it intravenously might face blood esterases, which could also inactivate the compound. It will be a matter galenics and pharmaceutical chemistry to inhibit the degradation of the peptide and find a proper application form and strategy for the drug. Chemical modification or even the development of nano particles could enable the drug to reach the organs in an active form.

Conclusion

The results of this thesis provide us with the suggestion, that AP318 enhances ENaC WT and three of the four investigated PHA1B α-stop-codon mutations cell surface expression with a maximal effect after 30 minutes. It already shows effects after 5 minutes, but to a smaller extent. The fourth mutant, αC63X, could not be detected at all. The gained knowledge contributes to our understanding of the effect of AP318 on ENaC cell surface presence, emphasising that AP318 does both: enhancing ENaC cell surface presence in western blot experiments as well as elevating the basal sodium current in ENaC WT and α-stop-codon mutants, measured in patch clamp experiments.

AP318 is a very good drug candidate for the treatment of ENaC loss of function related diseases such as PHA1B. We are heading towards an exciting future in which we are hopefully able to mitigate symptoms of patients with genetic malfunctions that cause phenotype diseases such as PHA1B

Index of abbreviations

APS - *ammonium persulfate*
AS - *amino acid*
ASIC - *acid-sensing ion channel*
bidest.H_2O - *bidestillated water*
BSA - *bovine serum albumine*
cAMP - *cyclic adenosine monophosphate*
CHO - *Chinese hamster ovaries*
Cryo-EM - *cryo electron microscope*
DMEM - *dulbecco's modified eagle medium*
DNA - *desoxyribonucleic acid*
ENaC - *epithelial sodium channel*
Erk - *extracellular signal-regulated* kinase
FBS - *fetal bovine serum*
g - *gravitational force*
GILZ1 - *glucocorticoide-induced leucine zipper*
GTP - *guanine triphosphate*
HAPE - *high altitude pulmonary edema*
HEK - *human embryonic kidney cells*
His - *6x histidine residues*
HRP - *horse raddish peroxidase*
IC50 - *half maximal inhibitory concentration*
LB medium - *lysogeny broth medium*
MR - *mineralocorticoid receptor*
MRE - mineralcorticoide response element
mRNA - *messenger ribonucleic acid*
NCC - *sodium chloride cotransporter*
Nedd4-2 - *neuronal precursor cell expressed developmentally down regulated protein 4-2*
NHS - *N-hydroxy succineimide*
NKCC - *sodium potassium chloride cotransporter*
nM - *nano molar*
OSR 1 - *oxidative stress responsive protein 1*
P2Y2 - *purinoreceptor 2*
PBS - *phosphate buffer saline*
PCR - *polymerase chain reaction*
PDK1/2 - *phosphoinositide-dependent kinase-1*
Pen/Strep - *penicilline/streptomycine*
PHA1A - *pseudohypoaldosteronism type 1A*
PHA1B - *pseudohypoaldosteronism type 1B*
PIP2 - *phosphatidylinositol 4,5-bisphosphate*
PIP3 - *Phosphatidylinositol-3,4,5-trisphosphate*
PKA - *proteinkinase A*
PY motif - *proline- tyrosine rich motif*
RAAS - *renin-angiotensin-aldosterone-systeme*
Raf - *rapidly accelerated fibrosarcoma*
ROMK - *renal outer medullary potassium channel*
SCNN1A - *sodium channel epithelial 1 α subunit*
SCNN1B - *sodium channel epithelial 1 β subunit*
SCNN1D - *sodium channel epithelial 1 δ subunit*
SCNN1G - *sodium channel epithelial 1 γ subunit*
SDS - *sodium dodecyl sulfate*
SGK1 - serum/ glucocorticoid regulated kinase 1
SPAK - STE20/SPS1-related proline/alanine-rich kinase
TEMED - *N,N,N',N'-tetramethyl ethylenediamine*
TIP - *TNF α lectin like domain derived peptide*
TM - *trans membrane domain*
TNF-α - *Tumor necrosis factor α*
TRIS - *tris(hydroxymethyl)aminomethane*
USP 2- 45 - *ubiquitin-specific protease 2-45*
WNK - *with no lysine kinase*
WT - *wild type*

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