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Glycosylation-dependent activation of epithelial sodium channel by solnatide

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ABSTRACT

Dysfunction of the epithelial sodium channel (ENaC), which regulates salt and water homeostasis in epithelia, causes several human pathological conditions, including pulmonary oedema. This is a potentially lethal complication of acute lung injury at least partially caused by dysfunctional alveolar liquid clearance, which in turn impairs alveolar gas exchange. Solnatide (named TIP-peptide, AP301), a 17 residue peptide mimicking the lectin-like domain of TNF has been shown to activate ENaC in several experimental animal models of acute lung injury and is being evaluated as a potential therapy for pulmonary oedema. The peptide has recently completed phase 1 and 2a clinical trials. In this study, we identify a glycosylation-dependent mechanism that preserves ENaC function and expression. Since our previous data suggested that the pore-forming subunits of ENaC are essential for maximal current activation by solnatide, we performed single- and multi-*N*-glycosylation site mutations in αN232,293,312,397,511Q- and δN166,211,384Q-subunits, in order to identify crucial residues for interaction with solnatide within the extracellular loop of the channel. Additionally, we generated αL576X and αN232,293,312,397,511Q,L576X deletion mutants of ENaC-α, since we have previously demonstrated that the carboxy terminal domain of this subunit is also involved in its interaction with solnatide. In cells expressing αN232,293,312,397,511Q,L576Xβγ-hENaC or δN166,311,384Q,D552Xβγ-hENaC activation by solnatide, as measured in whole cell patch clamp mode, was completely abolished, whereas it was attenuated in αL576Xβγ-hENaC- and δD552Xβγ-hENaC-expressing cells. Taken together, our findings delineate an *N*-glycan dependent interaction between the TIP-peptide and ENaC leading to normalization of both sodium and fluid absorption in oedematous alveoli to non-oedematous levels.

1. Introduction

The amiloride-sensitive epithelial sodium channel (ENaC), expressed at the apical side of polarised epithelia of the lung, distal colon, nephron, sweat and salivary glands, and other organs [1], comprises four types of homologous subunits, i.e. α,β, γ and δ [2–4]. The precise stoichiometry of these subunits remains elusive, but varies according to the tissue type and physiological conditions [5–9]. A functional channel is usually composed of one or two α- or δ-subunits together with a β- and a γ-unit, although channels made only of α-subunits or α–α or α–γ combinations can also conduct small currents [10]. ENaCs are members of the ENaC/degenerin (ENaC/DEG) family of ion channels, of which for one family member, the acid-sensing ion channel (ASIC), the structure has been experimentally determined [11,12]. ASIC-1 has a homotrimeric structure which strongly suggests that ENaC has to assemble as a heterotrimer in its biologically active state [13,14]. Each ENaC polypeptide chain is composed of short —NH_2 and —COOH termini located intracellularly and two transmembrane regions on either side of a large extracellular loop domain. Potential *N*-linked glycosylation sites vary in number between the subunits. As such,

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human α-ENaC contains seven sites, five of which lie in the extracellular loop, δ-ENaC has four sites, three of which are extracellular, whereas β-ENaC has nine sites all located extracellulary. Mutation of the *N*-glycosylation sites in rat α-ENaC does not affect channel activity [10,15], suggesting that trafficking and membrane insertion of the channel is independent of glycosylation of this subunit.

ENaC plays a critical role in Na^+ absorption and its dysfunction causes major disturbances in salt and water homeostasis. In the mammalian lung, regulation of Na^+ transport is crucial to maintain an optimal level of alveolar lining fluid necessary for efficient gas exchange [8]. Passive absorption of Na^+ through apically located ENaC and concomitant active transport through basolaterally-located Na^+/K^+-ATPase across the alveolar epithelial layer, results in osmotically-directed flow of water in the same direction, thus preventing flooding of the alveoli [16]. In some pathological conditions of the lung, such as acute lung injury and acute respiratory distress syndrome (ALI/ARDS), this mechanism is disrupted and pulmonary oedema ensues.

The synthetic cyclic peptide solnatide (also called TIP peptide AP301), which mimics the lectin-like domain of human TNF [17] is being developed as a therapy for pulmonary oedema. Solnatide activates both endogenously- and heterologously-expressed ENaC by increasing the open state probability, Po, of the channel [18–20]. The oligosaccharide-binding property of the lectin-like domain of TNF was suggested to play an important role in the mechanism by which TNF and solnatide interact with and activate ENaC, although the exact nature of this interaction is not yet understood. Lectin-like recognition of specific oligosaccharides, such as *N*,*N*′-diacetylchitobiose and branched trimannoses is characteristic of TNF and solnatide [17]. Blocking the lectin-like domain of TNF with *N*,*N*′-diacetylchitobiose, inhibited TNF-mediated increases in amiloride-sensitive membrane conductance of lung microvascular endothelial cells [21] and blunted activation of oedema reabsorption in an in situ flooded rat lung model [22]. Furthermore, PNGase F-mediated deglycosylation of A549 and H441 cells or of HEK-293 cells heterologously expressing human ENaC eliminated the Na^+ current-enhancing effect of solnatide in these cells, which is a strong suggestion for an interaction of TIP with carbohydrate groups on the extracellular loop of ENaC subunits [19,23].

The oedema reabsorption effect of the lectin-like domain of TNF is also lost upon mutation of residues T105, E107 and E110 of TNF or T6, E8 and E11 of AP301 to alanine [22,24] leading to the hypothesis that the hexapeptide sequence TPEGAE containing these residues is crucial for oligosaccharide binding (17). Mutant AP301 (A6, A8 and A11) has no effect on the amiloride-sensitive Na^+ current in A549 cells [18]. Furthermore, transgenic mice expressing a TNF mutant within the hexapeptide sequence (APAGAA) are more prone to develop lung oedema than their counterparts, when challenged with the bacterial toxin pneumolysin [23]. However, a mass spectrometry study of TIP peptide–carbohydrate complexes revealed that the mutant TIP peptide does still bind chitobiose albeit with a reduced affinity, as compared to that of the wild-type TIP peptide, suggesting that the hexapeptide sequence TPEGAE represents only a partial binding motif for the lectin-like activity of the TIP domain [25]. A computational study of oligosaccharide binding to solnatide failed to reveal a specific interaction via hydrogen-bonding between residues of the TPEGAE sequence and *N*,*N*-diacetylchitobiose or trimannose-*O*-ethyl [26]. Taken together these studies suggest that the TPEGAE sequence of the TIP domain is a partial oligosaccharide-binding motif in its interaction with ENaC, responsible for one step in a multi-step process which culminates in ENaC adopting a conformation with increased Po.

In the present study we aimed to determine which of the five predicted *N*-linked glycosylation sites in the extracellular loop of α-ENaC are important for solnatide-induced hENaC activation. To this end, the effect of solnatide on the amiloride-sensitive Na^+ current was evaluated in HEK-293 cells, which have no endogenous expression of ENaC and which heterologously express human ENaC subunits [27]. We introduced single and multiple mutations of the *N*-glycosylation sites of α- and δ-hENaC by site-directed mutagenesis. Endogenously-expressed α-, β-, γ- and δ-ENaC subunits in A549 and RPMI cells were also investigated quantitatively by Western blotting in the presence of solnatide.

2. Material and methods

2.1. Cell culture

Human alveolar epithelial A549 cells (ATCC no. CCL-185) in passages 80–97, and human embryonic kidney HEK-293 cells (ATCC no. CRL-1573) in passages 3–25, were seeded in Dulbecco's modified Eagle medium/F12 (DMEM/F-12, (Gibco™ by Life Technologies, LifeTech Austria) nutrient mixture Ham plus L-glutamine (Gibco™ by Life Technologies, LifeTech Austria), supplemented with 10% fetal bovine serum (FBS, (Gibco™ by Life Technologies, LifeTech Austria) and 1% penicillin–streptomycin (Sigma–Aldrich, Vienna, Austria). Cells were maintained at 37 °C with 5% CO_2 in a humidified incubator. After reaching confluence half of the medium was substituted with either DMEM/F-12 without FBS (control) or DMEM/F-12 without FBS containing solnatide, to reach a final concentration of 200 nM. Cell treatment was carried out in a time-dependent manner (5, 10, 30 min, respectively).

The cells were confluent monolayer (10^7 cells) at the time of harvesting. Medium was removed by suction. Cells were washed twice with phosphate buffered saline (PBS, (Gibco™ by Life Technologies, LifeTech Austria)), then harvested by scraping followed by re-suspension in extracting buffer (150 mM sodium acetate, 0.9% NaCl, 0.1% Triton X-100 pH 5.5), were ultrasonicated (3 × 10 s), and then incubated in ice for 30 min. The samples were cleared by centrifugation for 10 min at 13,000 rpm. Total protein concentration was determined by Bradford assay.

2.2. Site-directed mutagenesis

Enhanced green fluorescent protein tagged cDNAs encoding α-, β- and γ-human (*h*) ENaC were a kind gift from Dr. Deborah L. Baines (St. George's, University of London, London, UK). cDNAs encoding α-, β-, and γ-hENaC were a kind gift from Dr. Peter M. Snyder (University of Iowa, Carver College of Medicine, Iowa City, USA), and δ-hENaC was a kind gift from Dr. Mike Althaus (Justus-Liebig University, Giessen, Germany).

Point mutations were introduced into cDNA encoding α- or δ-hENaC using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA). Mutagenic primers were designed individually with the Primer Design Program provided on the producer's website and ordered from Sigma–Aldrich, Vienna,

Table 1
Mutagenic primers for glycosylation site mutations of α-hENaC, and C-terminal cut.

Mutant	Primer sequence (5′-3′)
α-N232Q	*Forward:* CTGTGCAACCAGCAAAAATCGGACTGCTTC
	Reverse: GAAGCAGTCCGATTTTTGCTGGTTGCACAG
α-N293Q	*Forward:* CTGCAACCAGGCGCAATACTCTCACTTC
	Reverse: GAAGTGAGAGTATTGCGCCTGGTTGCAG
α-N312Q	*Forward:* ATACTTTCAATGACAAGCAGAACTCCAACCTCTGG
	Reverse: CCAGAGGTTGGAGTTCTGCTTGTCATTGAAAGTAT
α-N397Q	*Forward:* GACTGCACCAAGCAAGGCAGTGATGTTC
	Reverse: GAACATCACTGCCTTGCTTGGTGCAGTC
α-N511Q	*Forward:* GATGCTATCGCGACAGAACCAGTACACCGTCAACAACAAGA
	Reverse: TCTTGTTGTTGACGGTGTACTGGTTCTGTCGCGATAGCATC
α-L576X	*Forward:* GCTCGTCTTTGACTAATAAGTCATCATGTTCCTC
	Reverse: GAGGAACATGATGACTTATTAGTCAAAGACGAGC