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A NOVEL TNF-MEDIATED MECHANISM OF DIRECT EPITHELIAL SODIUM CHANNEL ACTIVATION.

On line supplement

Methods.

Cells. Clara cell-like H441 lung adenocarcinoma cells were obtained from ATCC and were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES (pH 7.4), and 10% fetal bovine serum. We also used another lung cell model for patch clamp experiments. A highly transporting clone, 2F3, of the epithelial cell line A6, was a gift from Dr. Thomas Kleyman and was maintained by standard tissue culture techniques, as described previously (E1). For these cells, a culture medium consisting of a 50% (vol/vol) mix of DMEM and Ham's F-12 medium adjusted to appropriate tonicity plus 0.6% penicillin-1.0% streptomycin, 5% (vol/vol) fetal bovine serum, 1.5 µM aldosterone, 1 mM glutamine, and 25 mM NaHCO3 was used at 26 ^{o}C and 4% CO2. 2F3 cells were plated on permeable, glutaraldehyde-fixed, collagen-coated Millipore-CM filters (Millipore, Billerica, MA) attached to the bottoms of small Lucite rings at a density to allow them to be confluent and fully polarized after culturing for 10-14 days.

PLY Purification. LPS-free PLY was purified from a recombinant *Listeria innocua* 6a strain, expressing PLY in the laboratory of T.C. The batch of PLY used in this study had a specific activity of 1.25 ×10^{7} hemolytic units/mg.

Preparation of Na^{+}-K^{+}-ATPase containing fragments. ATPase-containing membrane fragments were prepared from the outer medulla of rabbit kidneys in the form of open membrane fragments using the procedure C of Jørgensen (E2). The enzyme activity

was determined by the linked pyruvate kinase/lactate dehydrogenase assay (E3). Na^+ affinity of the cytoplasmic binding sites was determined by a fluorescence assay using the styryl dye RH421 (E4).

ENaC-α transfection of H441 cells. Human sodium channel non-voltage-gated 1 alpha transcript variant 1 plasmid was purchased from OriGene (Rockville, MD). 70-80% confluent H441 cells were transfected with X-tremeGENE HP transfection reagent (Roche, In) according to the manufacturer's protocol, using a 2:1 ratio. After 48h, cells were collected, lysed and either tested by SDS-PAGE or used in *in vitro* pull-down assays.

Immunoprecipitation. ENaC-α transfected human lung adenocarcinoma epithelial cells grown in 60 mm culture flasks were washed with 1x PBS, scraped and lysed in 400 μl of 20 mM Tris-HCl, pH 7.4 buffer containing 0.15 M NaCl, 1%NP-40, 2mM EDTA, and protease inhibitors. The lysates (corresponding to 0.5 mg of total protein) were incubated with 0.1 μmole each of either biotinylated TIP peptide, biotinylated TIP peptide pretreated with *N,N′*-diacetylchitobiose/cellobiose and mutant TIP peptide coupled to MyOneTM streptavidin C1 magnetic beads (corresponding to about 100 μl of Dynabeads slurry) overnight at 4^oC. Afterward, beads were washed three times with 1x PBS and eluted in 150 μl of Laemmli buffer, upon which supernatants were analyzed by Western blotting.

In vitro GST Pull-Down Assay. Biotinylated TIP peptide, biotinylated TIP peptide pretreated with N,N′-diacetylchitobiose/cellobiose and mutant TIP peptide (0.5 nmol of peptide) were incubated with 0.3 nmole each of GST, GST–N-terminal domain, GST-C-terminal domain or GST-Extracellular loop ENaC-α fusion proteins, prepared as

described (E5), coupled to glutathione Sepharose beads (corresponding to 1/10 of Microspin column, GE Healthcare, about 60 μl of glutathione Sepharose slurry) for 1 h at 4^{o}C. Afterwards, the beads were washed and incubated with Streptavidin Alexa Fluor® 594 Conjugate for another 1 h at RT in dark. The complex was eluted in 200μl 10 mM Gluthatione elution buffer. Each eluent was transferred into a 96 well plate and measured the absorbance of triplicates at 600 nm wavelength against blank.

Whole cell voltage-clamped patch clamp experiments. Whole cell voltage clamped patch clamp experiments were performed in H441 cells, either pretreated for 5 min with 100 units N-glycosidase F (removal of N-linked oligosaccharides, Sigma, St Louis, MO) or not. Cells were perfused with Ringer's alone (ctrl) or with Ringer's containing either 50 μg/ml TIP or mutant TIP, in the presence or absence of amiloride (10 μM, Sigma, St Louis, MO). A voltage steps protocol was applied to vary the voltage in cells from – 140 mV to + 60 mV. The pipette solution was 135 mM K-gluconate, 10 mM KCl, 1 mM MgCl2, 10 nM NaCl, 5 mM glucose and 10 mM HEPES, pH 7.4 (1 N KOH). The bath solution's ionic composition was 140 mM NaCl, 2 mM CaCl2, 1 mM MgCl2 2.7 mM KCl, 10 mm HEPES, pH 7.4 (1 N NaOH).

Single-channel patch-clamp studies. Experiments were performed at room temperature using the cell-attached patch configuration as described (E6). Patch pipette and extracellular bath solutions consisted of a physiological 2F3 cell saline containing the following (in mM): 95 NaCl, 3.4 KCl, 0.8 CaCl2, 0.8 MgCl2, and 10 HEPES or 10 Tris, titrated with 0.1 N NaOH or HCl to a pH of 7.3–7.4. The TIP peptide and mutant TIP peptide were added to the apical or basolateral side of 2F3 cells cultured on glutaraldehyde-fixed, collagen-coated Millipore-CM filters (Millipore, Billerica, MA)

attached to the bottoms of small Lucite rings. Open probability (Po) of a single channel was calculated by dividing NPo by the number of channels in a patch. For our experiments, we determined mean Po for 10 min before addition of any agent and for 20 min after addition.

Rhodamine labeling of the TIP peptide. Rhodamine labeling was performed according to the manufacturer's protocol using Pierce® NHS-Rhodamine Antibody Labeling Kit (Thermo Scientific, Rockford, IL).

Immunofluorescence. H441 cells were plated onto glass coverslips and grown to 70-80% confluence for immunofluorescence. The uptake of the Rhodamine labeled TIP peptide was performed in HKR solution (5mM HEPES, 137 mM NaCl, 2.68 mM KCl, 2.05mM MgCl2.6 H2O, 1.8 mM CaCl2.2H2O, 1g/l Glucose) for 10, 30 and 60 min. The cells were then washed once with 1x PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4, pH 7.4) and fixed in 4% paraformaldehyde in 1x PBS for 10 min at room temperature. Coverslips were rinsed and mounted in ProLong Gold Antifade (Molecular Probes, Eugene, OR) and observed with a 63x objective on a Carl Zeiss microscope. Images were processed using Image J Imaging software. Single cell visualization were obtained collecting images with fluorescent structured illumination microscopy (SIM, Apotome.2, Axio Imager M2, Carl Zeiss) with a high magnification objective (oil 63X, NA: 1.4, lateral/axial resolution: ~ 240 nm). 4′,6-diamidino-2-phenylindole (DAPI) was used for nuclear staining.

Quantification of Rhodamine labeled TIP peptide uptake by fluorometry. 48h before the experiments, cells were seeded (100,000 cells/well) in 12-well plates. After they reached about 80% confluence, cells were incubated for 60 min at 37^{o}C with 5 □M

peptide solution prepared in HKR buffer. After removing the peptide-containing solution, cells were rinsed twice with HKR solution and trypsinized for 5 min at 37^{o}C. The peptides bound to the cell surfaces were thus removed by trypsin. After cells were detached they were suspended in HKR solution and collected by centrifugation. 0.1 M NaOH was used for at least 1h at 4°C to lyse the cells and the fluorescence of the lysates were measured at 552/575 nm.

Instillation i.t., capillary leak assessment and wet-to-dry ratio in vivo. Male mice were anesthetized with i.p. ketamine (150 mg/kg) and acetylpromazine (15 mg/kg) before the exposure of the trachea, PLY (1.5 µg/kg in saline) was instilled i.t. for 24 h in anesthetized mice via a 20-gauge catheter. For quantification of protein or pro-inflammatory mediators in BALF, collected BALF was centrifuged (500 g for 15 min at 4°C), supernatant was centrifuged again (5,000 g for 15 min at 4°C), and pure bronchoalveolar lavage fluid was used to measure total protein with the BCA Protein Assay Kit (Pierce). Absorbance was measured at 540 nm, and protein concentration was determined by using standard curves. Alternatively, cytokine/chemokine/growth factor concentrations were quantified with the multiplex MCYTOMAG-70K assay (EMD Millipore), according to the manufacturer's instructions. An Evans Blue dye (EBD)/Albumin mixture (30 mg/kg in saline) was injected into the tail vein 2h before mice were killed, in order to assess vascular leak. Lungs free of blood were weighed and snap-frozen in liquid nitrogen. The left lung was homogenized, incubated with formamide (18h at 60 °C), and centrifuged at 5,000 × g for 30 min. The optical density of the supernatant was determined spectrophotometrically at 620–750 nm. Extravasated EBD concentration in the lungs was calculated by using a standard curve

(μg of EBD per g of wet lung tissue). Lung wet-to-dry ratios were determined by weighing lungs immediately after isolation and then after drying them for 48 h at 65 °C in an oven.

Measurement of Phospholipase C activity. We used the EnzChek® Direct Phospholipase C Assay Kit (Molecular Probes, Life Technologies, Grand Island, NY), according to the manufacturer's instructions, to measure phosphatidylcholine-specific PLC activity.

Genotyping of the TKI mice offspring. Complete genomic DNA from the tail tip, extracted by using the DirectPCR Lysis Reagent (Viagen Biotech) was used to genotype the offspring by PCR. As a result of the previous cre-lox recombination, one loxP site with a size of 128 bp remained in the TNF gene locus of recombinant mice. Therefore, the primers TNF-945c (tag ttc aca ctc cac atc ctg ag) / TNF-7nc (caa gcc tgt agc cca cgt cg) spanning this region were used for the genotyping. In the case of wt mice the PCR reaction results in a fragment of 277 bp whereas the fragment received from homozygous k.i. mice had a size of 405 bp.

Results

Molecular Docking study between TIP peptide and ENaC-α.

In order to better identify the actual residues involved in the interaction between the TIP peptide within the C-terminus of ENaC-α, we have performed a molecular docking study. We followed the homology modeling approach, using the structure of the ENaC-related ASIC-1 channel as a template (E7). As shown in Figure E1, analysis of the extracellular part of ENaC-α predicted no binding sites for TIP peptide (presumably

because glycosylation was not modeled). However, using a reduced model of the carboxy-terminal part of ENaC-α lacking all amino acids beyond Leu584 (numbering according to the NP_001029.1 FASTA sequence), a binding site of a model of the TIP peptide on the carboxy-terminal α helix was localized, in agreement with our experimental data. The interaction is predominantly facilitated by the Arg of the TIP peptide that acts as a hydrogen bond donor and the Glu568 of ENaC-α. Less frequent binding occurs with yet another Glu (Glu571) and\or the trio of Val residues (Val563, Val566, Val 567). While the Glu568 and Glu571, as well as Val567, act as hydrogen bond acceptors from the Arg residue of the TIP peptide, the Val566 and Val563 accept hydrogen from the Gln residue of the peptide. The dominant hydrogen bond is however between the Arg of the TIP peptide and Glu568, with a distance between the donor and acceptor of 2.02 ± 0.17 Å and a binding energy of 19.3 ± 5.3 kJ/mol.

The interactions of the two molecules are further strengthened by their compatible shapes through the Van der Waals interactions of the amino acids at their respective contact areas. The contact area of the two molecules was 55 ± 16 $Å^2$. Here, the Glu of the TIP peptide binds within the groove formed by the kink in the α helix of ENaC-α's carboxyterminus, while the Arg provides stabilization of the TIP by hydrogen bonding on the sharp edge of the helix kink. The further stabilization may arise from interactions of Trp and the lone NH2 group of Cys with the Phe574 of ENaC-α. The binding energy, calculated from the difference of energies of the bound and unbound state summing the particular potential energies obtained from the force field, as well as the electrostatic and Van der Waals solvation energies between the TIP peptide and the carboxyterminal intracellular part of ENaC-α was estimated at 206 ± 37 kJ.mol-1. Using

the same calculation procedure, the mutant TIP peptide was found not to bind to ENaC-α. The domain in which the identified residues are situated in this molecular docking study was suggested to be involved in gating of ENaC (E8).

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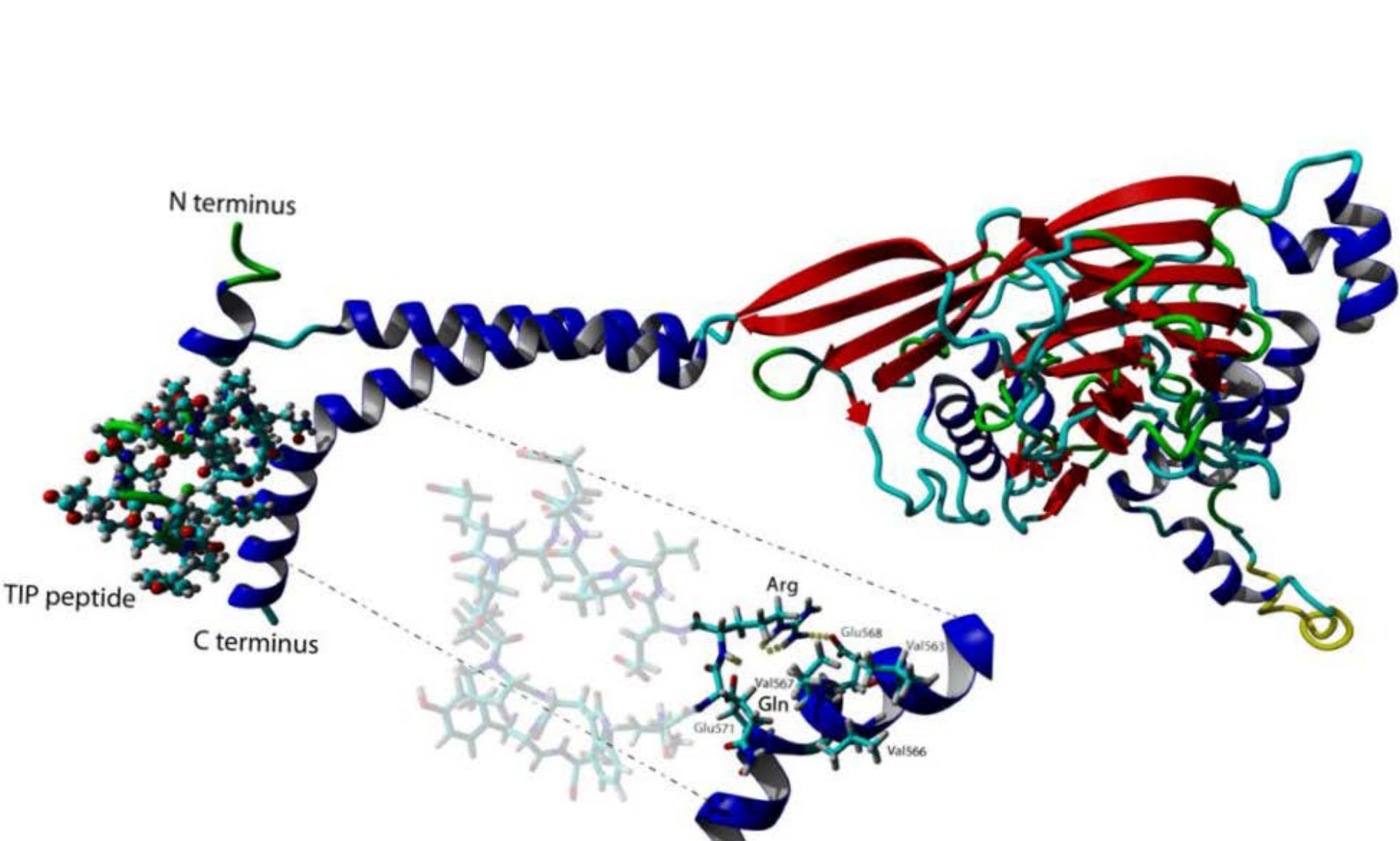
Legends.

Figure E1. Predicted interaction sites and residues between TIP peptide and a model of ENaC-α, based on the coordinates of the ASIC-1 channel (E7).


N terminus
TIP peptide
C terminus
Arg
Glu568
Val563
Val567
Gln
Glu571
Val566