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Mode of action of claudin peptidomimetics in the transient opening of cellular tight junction barriers

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ABSTRACT

In epithelial/endothelial barriers, claudins form tight junctions, seal the paracellular cleft, and limit the uptake of solutes and drugs. The peptidomimetic C1C2 from the C-terminal half of claudin-1's first extracellular loop increases drug delivery through epithelial claudin-1 barriers. However, its molecular and structural mode of action remains unknown. In the present study, >100 μM C1C2 caused paracellular opening of various barriers with different claudin compositions, ranging from epithelial to endothelial cells, preferentially modulating claudin-1 and claudin-5. After 6 h incubation, C1C2 reversibly increased the permeability to molecules of different sizes; this was accompanied by redistribution of claudins and occludin from junctions to cytosol. Internalization of C1C2 in epithelial cells depended on claudin-1 expression and clathrin pathway, whereby most C1C2 was retained in recyclosomes >2 h. In freeze-fracture electron microscopy, C1C2 changed claudin-1 tight junction strands to a more parallel arrangement and claudin-5 strands from E-face to P-face association – drastic and novel effects. In conclusion, C1C2 is largely recycled in the presence of a claudin, which explains the delayed onset of barrier and junction loss, the high peptide concentration required and the long-lasting effect. Epithelial/endothelial barriers are specifically modulated via claudin-1/claudin-5, which can be targeted to improve drug delivery.

1. Introduction

Regulation of solute movement through cellular barriers is a fundamental requirement for tissue compartmentation. In vertebrates, tight junctions (TJ) regulate the permeation of solutes across the intercellular space of neighboring epithelial and endothelial cells. TJs are a multi-protein complex, composed of different proteins, including the claudins (Clds) [1] and members of the TJ-associated MARVEL protein family, such as occludin (Occ) [2], marvelD3 [3] and tricellulin (Tric) [4]. Among the TJ-associated proteins, Clds have an outstanding role in selectively sealing the paracellular cleft against water soluble molecules and ions.

Clds are transmembrane proteins with cytosolic N- and C-termini, characterized by four transmembrane helices, as well as one intracellular and two extracellular loops (ECL1, ECL2). Structure-function studies have revealed that paracellular sealing is mediated by both ECL1 [5] and ECL2 [6]. It is assumed that direct interaction of the ECLs of Clds from adjacent cells, so-called *trans*-interaction, is responsible for selectively tightening the intercellular space [7]. Since the 27 known Cld family members [8] display different paracellular charge and size selectivity, the barrier properties of a given tissue or cell layer are directly influenced by the specific Cld expression pattern [9]. Knockout experiments have shown that the loss of Cld5 from the blood-brain barrier [10] or Cld1 from mammalian skin [11] leads to breakdown in paracellular sealing and consequently increases intercellular permeability. Moreover, several diseases can be linked to mutations [12] or modified expression patterns of specific Clds [13].

These findings highlight the important role of Clds for creating compartmentalization and maintaining homeostasis of these compartments by selectively paracellular solute flux. On the other hand, many hydrophilic drugs exhibit very limited or virtually no

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permeation through, e.g. blood-neural barriers [14,15], due to Cld-mediated sealing. Consequently, modulation of Cld interactions to raise paracellular penetration of pharmaceutically active compounds is a promising strategy to improve drug delivery. Several studies have been carried out to transiently modulate TJ interactions [16], whereby TJ protein-derived peptides are able to modulate their parent protein. For example, a Cld-derived peptide has been successfully used to open the paracellular barrier [17]. Another Cld1 peptidomimetic (C1C2) can reversibly open the peripheral nerve barrier (PNB) *in vivo*, allowing PNB-impermeable drugs to penetrate this barrier within three days of peptide administration [18]. Moreover, it has been shown that C1C2 colocalizes with Cld1 and reduces the expression and localization of Cld1 at the cell membrane. Nevertheless, the mode of action of this peptide remains unclear at the cellular level.

The aim of the present investigation was to clarify whether C1C2 influences other Clds than Cld1 and if so, whether there are differences between the Clds with respect to their sensitivity to C1C2. Furthermore, we wanted to characterize the time dependency of the permeability increase caused by C1C2, in order to elucidate its action profile. The data obtained indicate that C1C2 affects the localization of Cld1-5 and Occ, whereby C1C2 preferentially interacts with Cld1 and -5. C1C2 redistributes Cld1-5 and Occ from the cell membrane to the cytosol and leads to reorganization of TJ-strand structure, thereby increasing intercellular permeability. The late onset of barrier opening, starting at about 6 h after administration, is explained by the steric hindrance of Clds within TJ strands, as well as the prolonged presence of C1C2 in recycling endosomes, which keeps the peptide away from sites where it can regulate TJ function.

2. Materials and methods

2.1. Peptide design and synthesis

Peptides were synthesized automatically by using Fmoc chemistry as described previously by Ref. [18]. Experiments were performed with the Cld1-derived peptide C1C2 (SSVSQSTGQIQSKVDSLLNLNSTQATR-NH_2) and, as a control, the Cld2-derived peptide C2C2 (ESATHSTGITQSDIYSTLLGLPADIQAAQ-NH_2). The two peptides represent the C-terminal half of the ECL1 of mouse Cld1 and -2, respectively, from amino acid positions 53 to 81. Peptides were amidated at the C-terminus and cysteines Cys^{54} and Cys^{64} were replaced by serines. The peptides were visualized by N-terminal labeling with 5,6-carboxytetramethylrhodamine (TAMRA), resulting in TAMRA-C1C2 or -C2C2.

2.2. Cell culture

Human intestinal epithelial cells (Caco-2) were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Darmstadt, Germany), including 20% fetal calf serum (FCS; Life Technologies), 100 units/ml penicillin, 100 μg/ml streptomycin (Biochrom, Berlin, Germany) and 1x non-essential amino acids (NEAAs, Life Technologies). Human embryonic kidney cells (HEK)-293 and Madin-Darby canine kidney cells (MDCK-II) were cultured in DMEM as Caco-2, but with 10% FCS and without NEAAs. Cells were cultured at 37 °C and 5% (Caco-2) or 10% (HEK-293, MDCK-II) CO_2. Primary mouse brain microvascular endothelial cells (pMBMECs) were isolated from 7 to 9 week-old C57BL/6 mice (Harlan Laboratories, The Netherlands) and cultured as described [19,20] in DMEM supplemented with 20% FCS, 1 mM sodium pyruvate (Life Technologies), 1% NEAAs, 50 μg/ml gentamycin (Life Technologies), and 1 ng/ml basic fibroblast growth factor (Life Technologies) and grown on Matrigel-coated filter inserts (0.4 μm pore size, 6.5 mm diameter, Transwell® Costar, Corning, The Netherlands; Greiner Bio-one, Germany). Confluent pMBMECs were used from day 6 to day 8 after isolation.

2.3. Transepithelial electrical resistance (TER) and permeation measurements

For peptide treatment of Caco-2 or MDCK-II monolayers, cells were grown on rat tail collagen-coated filters (0.4 μm pore size, 10 mm inner diameter, Millicell-CM; Millipore, Eschborn, Germany) with media exchange every 1-2 days. After 2 days (MDCK-II) or 5-7 days (Caco-2) of culture, medium was replaced by medium without or with C1C2 or C2C2 (30-300 μM). Medium with peptide was administrated either only in the apical compartment or in both compartments. TER was measured manually with the EVOM voltohmmeter (World Precision Instruments, Sarasota, USA). During measurement, 24-well plates with the filters were placed on a 37 °C heated plate [18]. For peptide treatment of pMBMECs, cells were grown on Matrigel-coated filter inserts for 6 days and, then, apically exposed to medium with or without either C1C2 (200 μM) or C2C2 (200-300 μM). TER was automatically monitored using a cellZscope (nanoAnalytics, Münster, Germany).

Twenty four hours after treatment, the permeation of differently sized molecules (e.g., lucifer yellow, LY, 457 Da; 10 kDa fluorescein isothiocyanate-dextran, FD10; Sigma–Aldrich, Taufkirchen, Germany and 3 kDa AlexaFluor 680-dextran, AD3; Life Technologies, Darmstadt, Germany) from the apical to the basolateral filter sides was measured to calculate paracellular permeability coefficients (P). For Caco-2 and MDCK-II cells the apparent permeability coefficient (Papp; [6]) was calculated by the following equation: $Papp = dQ/dt \cdot (1/A \cdot C_0 \cdot 60)$ in cm/s, where dQ/dt is the permeability of the tracer (μg/min), A is the surface area of the filter (cm^2), C_0 is the concentration of the tracer in the apical compartment (μg/min). Cell culture plates with Caco-2 and MDCK-II on filters were placed on a 37 °C heated plate and washed 2× with preheated Hank's buffered salt solution with Ca^{2+} and Mg^{2+} (HBSS+/+; Life Technologies). Subsequently, 400 μl of preheated 45.7 μg/ml LY or 25 μg/ml FD10 in HBSS+/+ were added apically. The basolateral compartment was filled with 600 μl HBSS+/+. Over the next 30 min, samples were taken from the basolateral compartment, whereby cells were incubated at 37 °C and 5% (Caco-2) or 10% CO_2 (MDCK-II). LY and FD10 samples were measured using the microplate reader Tecan Infinite M1000 Pro (Tecan, Group Ltd, Männedorf, Switzerland). For permeability measurements with pMBMECs, inserts were rinsed with assay medium (HBSS+/+ supplemented with 5% FCS and 25 mM Hepes, pH7.2 - 7.5) and transferred to 24-well plates containing 0.6 ml of assay medium per well. The apical medium of the filters was removed and replaced by 0.1 ml of assay medium containing AD3 (10 μg/ml) or LY (22.9 μg/ml). At different time points, inserts were placed in another well, containing 0.6 ml of assay medium. Incubations were performed at 37 °C. To calculate the endothelial permeability coefficients (Pe) of AD3 and LY in pMBMECs, the average volume cleared was plotted versus time and the slope was estimated by linear regression analysis. The slope corresponds to the PSt while PS represents the permeability surface area product (ml/min). The slope of the clearance curve with the control insert (filter coated without cells) was denoted PSf. The PS value for the cell monolayer (PSe) was calculated from: $1/PSe = 1/PSt - 1/PSf$. The PSe value was divided by the surface area of the inserts to generate the endothelial permeability Pe (cm/s). The fluorescence intensity of AD3 was quantified using the Odyssey Infrared Imaging System (LI-COR, Bad Homburg, Germany). Subsequently to the permeation experiments, filters were analyzed by phase contrast microscopy to exclude damage of the cell monolayer during TER and permeation experiments.

2.4. Immunocytochemistry

Immunocytochemistry of Cld1 to -5 and Occ was performed on filters with Caco-2 and MDCK-II cells washed 2× with Dulbecco's phosphate-buffered saline with Ca^{2+} and Mg^{2+} (DPBS+/+; Biochrom). Ice cold 99% ethanol was added to the apical and basolateral compartments and incubated for 10 min at -20 °C. After washing the filters (2× DPBS+/+), filter membranes were cut out and incubated for 10 min with 0.1% Triton X-100 (Sigma-Aldrich) in DPBS+/+, followed by washing (4× DPBS+/+). Cells were blocked with 5% FCS in DPBS+/+ for 1 h at room temperature. Cells were incubated with primary antibody mixtures of mouse anti-human Occ (2.5 μg/ml, #33-1500) together with 1.25 μg/ml of either rabbit anti-human Cld1 (#51-9000), rabbit anti-human Cld2 (#51-6100), rabbit anti-mouse Cld3 (#34-1700), rabbit anti-mouse Cld4 (#36-4800), rabbit anti-mouse Cld5 (#34-1600) or rabbit anti-human Tric (#70-0191) (all Life Technologies) in DPBS+/+ over night at 4 °C. After washing (4× DPBS+/+), the secondary antibodies Alexa Fluor 488 goat anti-rabbit (8 μg/ml, #A-11029) and Cy3 goat anti-mouse (8 μg/ml, #A-10521) (Life Technologies) were added together for 1 h at room temperature. After washing (4× DBPS+/+), the filter membranes were put on a microscope slide (Roth, Karlsruhe, Germany) and mounted with Immu-Mount (Thermo Fisher Scientific, Schwerte, Germany). Samples were analyzed using the Laser Scanning Microscope 510 META-NLO (Zeiss, Jena, Germany) and Zeiss LSM Image Browser Software (Zeiss).

pMBMECs were fixed with ice cold methanol for 30 s, then rinsed in 1× DPBS-/- and stained for rabbit anti-mouse Cld3 (#34-1700), rabbit anti-mouse Cld5 (#34-1600) and rabbit anti-mouse Occ (#71-1500) (Life Technologies). Unspecific binding was blocked by incubating the cells with 1× Tris buffered saline (TBS) containing 5% skimmed milk and 0.2% Triton X-100 for 30 min. Primary antibody (10 μg/ml) was diluted in 1× TBS containing 5% skimmed milk and 0.2% Triton X-100 and incubated for 30 min at room temperature. After washes with DPBS-/-, the secondary antibody (Alexa Fluor 488 goat anti-rabbit, 10 μg/ml, #A-11034; Life Technologies) was added for 30 min at room temperature in 1× TBS containing 5% skimmed milk and 0.2% Triton X-100. After washing (1× DPBS-/-), samples were mounted using Mowiol (Sigma–Aldrich) and analyzed using a Nikon Eclipse E600 microscope connected to a Nikon Digital Camera DXM1200F with the Nikon NIS-Elements BR3.10 software (Nikon, Egg, Switzerland). Images were processed and mounted using Adobe Photoshop software (Adobe Systems, CA, USA).

2.5. Internalization and inhibitor studies

HEK-293 cells stably expressing Cld1-yellow fluorescent protein (Cld1-YFP) [21] were cultured on poly-L-lysine (Sigma-Aldrich) coated cover slips until they reached a cell density of about 85%. TAMRA-C1C2 was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and added to medium containing 0.012% Pluronic F127