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Claudins in the tight junctions of stria vascularis marginal cells

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Abstract

In the mammalian cochlea, tight junctional strands are visible on freeze fracture images of marginal cells and other inner ear epithelia. The molecular composition of the strial tight junctions is, however, largely unknown. We investigated the expression of integral tight junction-proteins, claudin-1 to -4, and occludin, in stria vascularis of the guinea-pig cochlea, as compared to kidney. Western blot analysis revealed a strong expression of claudin-4 and occludin in strial tissue, and confocal immunofluorescence microscopy demonstrated their presence in the tight junctions of the marginal cells. In addition, a moderate level of claudin-3 and claudin-1 was detected and both were located in the marginal tight junctions. Claudins-1, -3, and -4 are characteristic of epithelia with low paracellular permeability and claudin-4 is known to restrict the passage of cations through epithelial tight junctions. In the marginal cells, these claudins appear to be responsible for the separation of the potassium-rich endolymph from the sodium-rich intrastrial fluid. In contrast, Western blot analysis and confocal microscopy demonstrated that the marginal cell epithelium does not contain claudin-2, which forms a cation-selective pore in tight junctions. Its absence indicates a cation-tight paracellular pathway in the marginal cells.

Keywords: Cochlea; Inner ear; Guinea pig; Occludin; Zonula occludens; Epithelium; Barrier function

Tight junctions seal the paracellular pathway of epithelia. They comprise the integral transmembrane proteins junctional adhesion molecule [1], occludin [2], and members of the claudin multigene family [3]. While the functional roles of junctional adhesion molecule and occludin are not yet clear [4], claudins have been shown to control the permeability of the paracellular pathway. When expressed in fibroblasts, claudins by themselves form the circumferential strands that are characteristic of tight junctions [5]. Claudins are found in vertebrates only [6]. In mammals, the claudin family of ~23 kDa integral membrane proteins includes at least 24 members [7].

Experiments with kidney epithelial (MDCK) cells demonstrated that claudins are involved in the regulation of conductance and ionic selectivity of the paracellular pathway. Clones of MDCK cells with high and low transepithelial resistances express claudin-1/-4 and claudin-1/-2/-4, respectively [8]. Overexpression of claudin-4 in low-resistance MDCK cells causes a selective decrease in the permeability of small cations (Na^+, K^+) without affecting Cl^- permeability or the flux of uncharged solutes [9]. By contrast, if claudin-2 is transfected and expressed in a high-resistance MDCK clone, a formation of paracellular pores leads to a decrease of transepithelial resistance and a selective increase in cation permeability [10].

Electron microscopical investigations showed tight junctions in marginal and dark cells, which form the secretory epithelia of the cochlea and of the vestibular organ, respectively [11]. Morphologically, the tight junctions of sensory and stria vascularis epithelia are described as very tight with respect to the number of strands visible in freeze fracture images. These epithelia contain more strands than others lining the endolymphatic space [12]. At the apical side of the marginal cells and in basal cells, the intracellular, tight

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junction-associated protein ZO-1 was found [13]. The integral tight junction-protein claudin-14 was not found in the stria vascularis [12]. Little else is known, however, about the tight junctions of marginal cells.

In the present study, we investigated the expression of integral tight junction-proteins, claudin-1 to -4 and occludin, in the stria vascularis of guinea pig cochlea and visualized them in the tight junctions of the marginal cells.

Materials and methods

Tissue preparation. Healthy, pigmented, adult guinea pigs of both sexes with intact Preyer's reflex were anesthetized and subsequently killed by inhalation of 100% CO_2. The cochleae were opened and the bony walls were micromechanically removed as described previously in detail [14]. The stria vascularis of each cochlear turn was transferred to a cell-tak (BD Biosciences, Bedford, USA) coated coverslip in a culture dish (35 mm diameter; Nalge Nunc International, Denmark). With the culture dish on an inverted microscope, explant debris of non-strial tissue was removed.

In addition to stria vascularis, a tissue sample from the guinea pig kidney, containing cortical and medullary tissue, which means that the cells comprise mostly proximal tubule epithelia because of their large abundance, was taken and stored at −80 °C. Furthermore, distal colon was excised, opened along the mesenteric insertion, and rinsed with Ringer's solution. Submucosal tissue was removed by stripping [15], leaving epithelium and lamina propria intact. These samples were also stored at −80 °C before Western blot analysis.

Immunofluorescence microscopy. For fluorescent imaging of tight junction proteins, tissues were stained according to the following protocol (if not stated otherwise, the preparations were performed at 37 °C).

After mounting on coverslips, the tissues were washed twice with phosphate-buffered saline (PBS) and then fixed with methanol at −20 °C for 15 min. After washing them again with PBS, the tissues were permeabilized with 0.5% Triton X-100 for 10 min at room temperature. To block non-specific binding sites, tissues were then bathed in PBS containing 0.5% (v/v) goat serum (blocking solution) for 10 min at room temperature. All following washing procedures were performed with this blocking solution. Antibodies (Zymed Laboratories, San Francisco, USA) used for immunostaining included: claudin-1 to -3 polyclonal antibodies, claudin-4 monoclonal antibody, and anti-human occludin monoclonal or polyclonal antibodies, respectively (all were diluted 1:50 in blocking solution). For claudin-staining, the tissues were incubated for 60 min and, after two washes, tissues were incubated with antibodies against occludin for 30 min. After two additional washes, cells were incubated with Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, USA, diluted 1:500 in blocking solution) for 30 min and washed twice before incubating with Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes, USA, diluted 1:500 in blocking solution) for another 30 min. After two washes the nuclei were stained with the DNA-specific fluorochrome 4′,6′-diamidino-2′-phenylindoladihydrochloride (DAPI, Boehringer Mannheim, Germany), diluted according to the manufacturer's instructions, for 30 min, and washed again before mounting in ProTags MountFluor (Biocyc, Luckenwalde, Germany). Fluorescence images were obtained with a confocal laser scanning microscope (LSM510, Carl Zeiss Jena GmbH, Jena, Germany).

Western blot analysis. Tissues were homogenized by douncing in iced lysate buffer containing 20 mmol/L Tris, pH 7.4, 5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.3 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 μL/mL aprotinin, 16 μg/mL benzamidine HCl, 10 μg/mL phenanthroline, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 1 mmol phenylmethylsulfonyl fluoride, 210 μg/mL sodium fluoride, 2.16 mg/mL β-glycerophosphate, 18.5 μg/mL $NaVO_4$, and 1 μl/mL trypsin inhibitor (all substances obtained from Sigma Chemical, St. Louis, MO). Membrane fractions were obtained by freeze–thaw cycles and subsequent passage through a 26 G × 1/2-in. needle. To remove insoluble material, the extract was centrifuged at 200*g* for 5 min at 4 °C. The supernatant was then centrifuged at 43,000*g* for 30 min at 4 °C. The pellet was resuspended in lysate buffer and protein content was determined using BCA Protein assay reagent (Pierce, Rockford, IL) and quantified with a plate reader (Tecan, Austria). Aliquots of 7.5 μg protein of pooled tissues of at least 3 animals were separated by polyacrylamide gel electrophoresis (10%) and transferred to a blotting membrane (NEN Life Science Products, Boston, MA). For detection of claudin-4 with a monoclonal antibody, 10 μg protein was used.

All blots were blocked for 2 h in 5% milk powder and subsequently in 5% bovine serum albumin (at 4 °C) overnight before incubation with primary polyclonal antibodies (claudin-1 to -3 and occludin) or monoclonal primary antibodies (claudin-4). Secondary antibodies were peroxidase-conjugated goat anti-rabbit polyclonal immunoglobulin G (for claudin-4 also a goat anti-mouse monoclonal immunoglobulin G antibody). Chemiluminescence was induced with a Lumi-LightPLUS Western blotting kit (Roche, Mannheim, Germany), detected using LAS-100 imaging system (Fuji, Tokyo, Japan), and analyzed with quantification software (AIDA, Raytest, Berlin, Germany). Because of the little amount of protein from the stria samples, the densitometric analysis was performed only with the western blots shown.

Antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA). Specificity of these antibodies was tested by analyzing tissue samples from colon and kidney of the guinea pigs because the claudins and occludin under investigation are expressed in mammalian kidney [16,17] and distal colon (except claudin-2) as well [18].

Results

Tight junction protein expression

Immunoblot analyses were performed for the tight junction-proteins claudin-1 to -4 and occludin. Crude membrane fractions were used because these proteins are integral membrane proteins forming tight junction strands. All claudins were detected as a band at ~23 kDa (Fig. 1B). The protein expression of the kidney sample served for reference (100%) since all of the claudins studied are expressed in different nephron segments as well [16].

Western blot analysis (Figs. 1A and B) and subsequent densitometry (Fig. 1C) showed that the expression of occludin and claudins was smaller in stria vascularis than in kidney. The amount of occludin in the stria vascularis was 57% of that in kidney. Immunoblotting of protein extracts from the kidney revealed two main bands with apparent molecular weights ranging from 55 to 65 kDa. In stria vascularis, only one band was detected with an apparent molecular weight of about 65 kDa.

Claudin-1 and -3 showed moderate levels in the stria vascularis, with 20% (claudin-1) and 43% (claudin-3) of the expression in kidney, respectively. Claudin-4 was

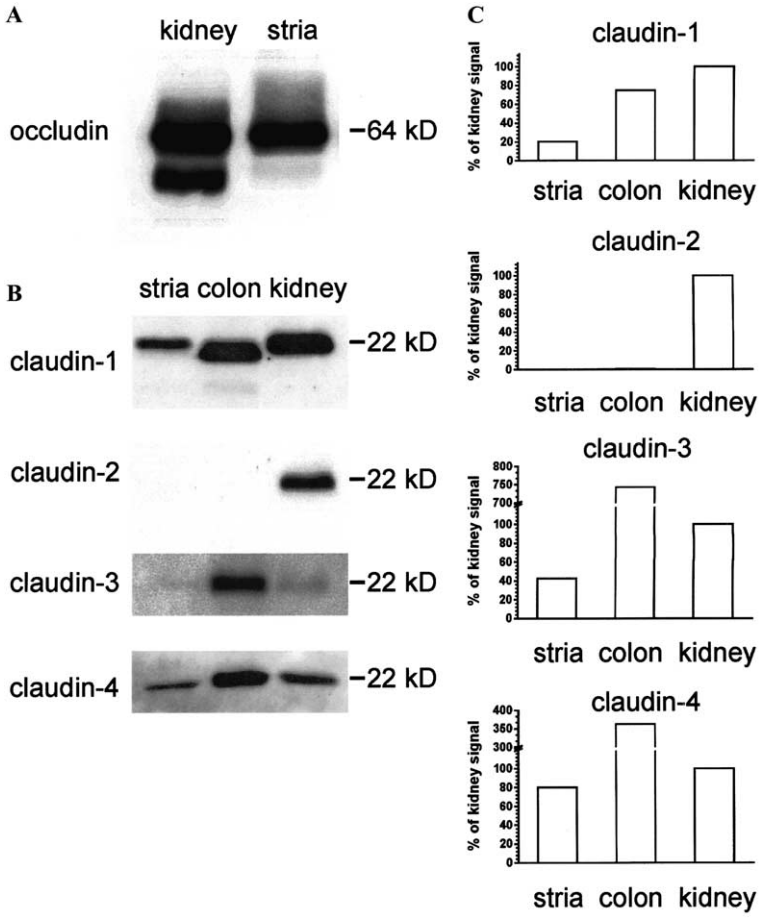

Fig. 1. Western blot analysis of occludin and claudin-1 to -4. (A) Western blot analysis of membrane fractions of stria vascularis and kidney samples showed occludin expression in both tissues. In contrast to the stria vascularis, immunoblotting of protein extracts from the kidney revealed two main bands with apparent molecular weights ranging from 55 to 65 kDa. (B) All claudins were expressed in the kidney. Expression of claudin-1 was similar in colon and kidney, but much lower in the stria. No claudin-2 was detected in the stria and colon. Claudin-3 was detected in stria vascularis samples and had a strong expression in the colon. With antibodies against claudin-4, strong expression was observed in all tissues. (C) Densitometric analysis of Western blots compared to kidney signals, which were set as 100%. Claudin-1, -3, and -4 showed expression levels of 20%, 40%, and 80%, respectively. In contrast, claudin-2 was not detectable in the stria vascularis and in the colon.

stronger expressed, i.e., it was found to be at a level of 80% compared to kidney (Figs. 1B and C). In contrast to the other three claudins investigated, claudin-2 was not detectable in the stria vascularis, although markedly expressed in the kidney, which mainly consists of proximal tubular epithelia. (Figs. 1B and C).

We also investigated a rather tight epithelium, distal colon, where the paracellular permeability is small [19]. The pattern of tight junction proteins was found to be similar to what we could demonstrate in stria vascularis, i.e., claudin-1 was at a lower level (75%) whereas claudin-3 (743%) and claudin-4 (362%) showed stronger signals than the kidney samples. The tight junction molecule claudin-2 was not detectable.

Immunofluorescent studies

Confocal immunoflourescent microscopy was performed to characterize the subcellular distribution of occludin and claudins in stria vascularis' marginal cells (Fig. 2). In parallel to the epithelium we scanned the *xy*-plane (square image). *xz*-Projections were computed at the indicated green line (rectangular image on top of the square image), and *yz*-projections at the indicated red line (rectangular image to the right of the square image). Evidence for tight junction proteins was found only in the *xy*-scans focussed on (a) the plane of marginal cells' tight junctions (Figs. 2, 1–3) and (b) a parallel plane below, probably at the basal cells' tight junctions (Figs. 2, 4–6). The confocal images revealed a complete epithelial network of epithelial tight junctions in the marginal cells (Figs. 2, 1–3) of the stria vascularis. Staining of basal cells' tight junctions was sporadic and much weaker (Figs. 2, 4–6). The weaker DAPI signals from the deeper tissue layers of the stria vascularis suggest that diffusion of the dyes to cells below the marginal cells was impaired, resulting in a weaker staining of basal cells' tight junctions. The incoherent staining

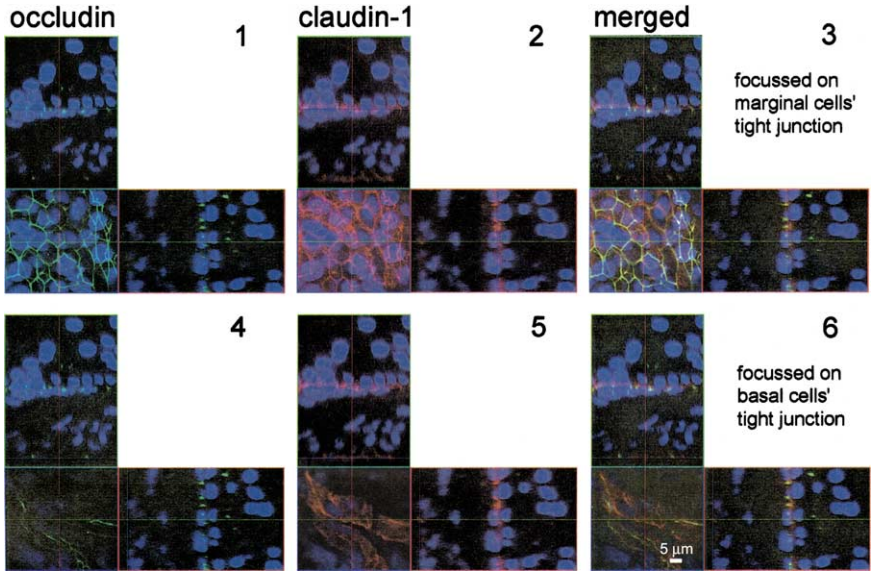

Fig. 2. Immunofluorescent staining of the stria vascularis. Stria vascularis was mounted on a coverslip and stained with a monoclonal antibody against occludin (green) and a polyclonal antibody against claudin-1 (red). Nuclei were stained with DAPI. We computed an *xz*-projection at the indicated green line (rectangular image on top of the quadratic image), and a *yz*-projection at the red line (rectangular image to the right of the quadratic image). The *xy*-scans shown were focussed at the plane of marginal cells' tight junctions (1–3), and at a parallel plane below, probably on the basal cells' tight junctions (4–6).

suggests that parts of the basal cells may be destroyed when the stria vascularis was micromechanically removed from the bony walls of the cochlea. However, in the Western blot analysis, tight junction proteins of basal cells may contribute to the membrane fraction of stria vascularis samples.

The *xy*-scans focussed on the plane of marginal cells' tight junctions delineated a complete network of tight junctions (Fig. 3). Superposition of confocal images demonstrated a colocalization of claudin-1 and claudin-3 to -4 with occludin (Fig. 3), whereas no claudin-2 expression was detectable in marginal cells in accordance with Western blot analysis (Fig. 3, second panel).

Discussion

In the cochlea, the barrier function of epithelial tight junctions is essential for sensory transduction and the compartmentalization of the fluid-filled spaces of the cochlea. Thus, mutation of the tight junction-protein claudin-14 can lead to hereditary deafness DFNB29 [12]. There are, however, only few reports on integral tight junction proteins of the mammalian inner ear. In cultured epithelial cells from semicircular canals (without receptor organs), the intracellular tight junction-associated protein ZO-1 as well as the integral tight junction-protein occludin were found [20]. Recently, the integral tight junction-protein claudin-14 was found in various epithelial cells of the cochlear duct, but not in the stria vascularis.

Epithelial barriers play a key physiological and pathophysiological role in the cochlea. The cochlear duct contains endolymph, a unique extracellular fluid, which is high in K^+ and low in Na^+ [21]. One of the tissues delineating the endolymphatic space is the stria vascularis. Impairment of stria vascularis permeability may lead to different types of so-called 'sensorineural deafness' ("endolymphatic deafness") [22]. The stria vascularis contains three special cell types and two epithelial barriers [23]. Facing the endolymph, the strial marginal cells form an epithelium which separates the endolymph from intrastrial fluid.

The ionic composition of this intrastrial fluid resembles that of the perilymph, being high in Na^+ and low in K^+ [24,25]. On the opposite side of the stria vasularis, the basal cells form an epithelium which separates the intrastrial fluid from perilymph. Intermediate and basal cells of the stria vascularis are part of the gap-junctional pathway for K^+ cycling of the cochlea [26]. K^+ is released from the intermediate cells into the intrastrial space, thus, generating the endocochlear potential [27]. Tight junctions are formed in the stria vascularis before the development of the high potassium concentration in the endolymph [11]. The unusual Na^+/K^+ asymmetry of the extracellular fluids separated by the marginal cells definitely requires that the paracellular pathway of the marginal cells must form a cation-tight barrier.

To elucidate the molecular basis of the barrier function, we analyzed the expression of integral tight junction proteins and imaged their distribution in the stria vascularis marginal cells.

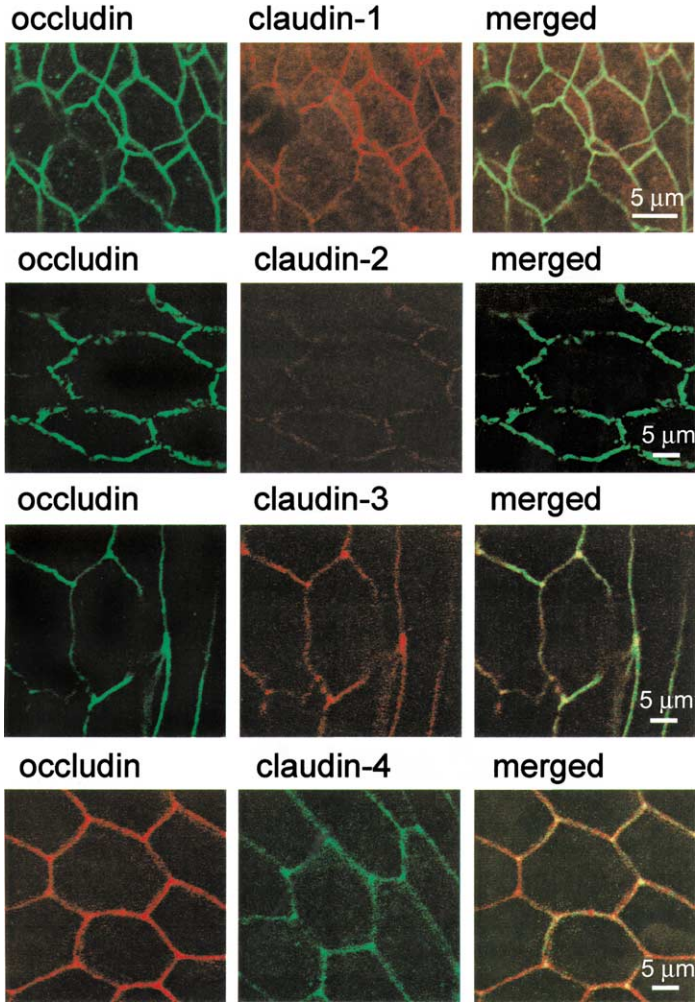

Fig. 3. Occludin and claudin-1 to -4 in stria vascularis marginal cells. Immunofluorescent staining with polyclonal or monoclonal antibodies against the transmembrane tight junction proteins occludin and claudin-1 to -4. Occludin and claudin-1, -3, and -4, but not claudin-2, were detected in the stria vascularis marginal cells' tight junctions.

Unlike the claudin gene family consisting of more than 20 closely related members [28], there is only one occludin identified. In MDCK cells, an occludin splice variant is characterized by an alternative N-terminal sequence [29]. Subcellular localization of this variant at the tight junction is not different compared to the originally described occludin [30]. Recently, the existence of at least four different forms of occludin molecules resulting from alternative splicing was shown to exist in the colonic cell line HT-29/B6 [31]. Because of the crude membrane preparation there could also be a proteolytic cleavage of occludin resulting in an occludin pattern with more than one band. The observed occludin pattern in Western blots of our kidney samples can be explained by posttranslational modifications or an additional splice variant, whereas in the stria vascularis only one form exists.

Our Western blot analysis showed that the integral tight junction protein claudin-4 is strongly expressed in the stria vascularis. Confocal immunofluorescence microscopy demonstrated that claudin-4 is located in the tight junctions of the marginal cells. Since claudin-4 decreases Na^+- and K^+-permeability without affecting Cl^- permeability [9], presence of this protein in the marginal cells' tight junctions suggests a barrier against cations. This is in line with the physiological importance of marginal cells as outlined above.

In our series, moderate levels of claudin-3 and claudin-1 were found. Both proteins were localized in the marginal tight junctions. Analysis of different tissues, e.g., in the nephron [16], provides evidence that claudin-1, -3, and -4 are a characteristic feature of epithelia with low paracellular permeability. Although details of the paracellular ion selectivity of claudin-1 and -3 are not yet known, the present findings suggest that claudin-1 and -3 contribute to the barrier function of marginal cells.

Claudin-3 and -4 are among the genes that map within the Williams–Beuren syndrome common deletion

[32]. It is not known whether the endolymph is ionically altered in this disease. It is conceivable, however, that impairment of the paracellular barrier in the marginal epithelium of the stria may result in changes of ionic composition or electrical potential of the endolymph. This hypothesis is clinically challenging because children with Williams–Beuren syndrome are described to suffer from high-frequency sensorineural hearing loss and to be more susceptible to acute hearing impairment [33].

Claudin-2 was not detected in the stria vascularis and was not found in the tight junctions of the marginal cells. Another tight epithelium, distal colon, also showed no expression of claudin-2. Claudin-2 forms cation-selective pores [8,10]. Hence, absence of claudin-2 in the marginal tight junctions is necessary for a barrier against cations.

In conclusion, our results of a strong expression of claudins-4, a moderate level of claudin-3, -1, and absence of claudin-2 are consistent with the putative barrier function of the marginal cells in stria vascularis of the mammalian cochlea.

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