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A NEW MODEL OF OCCLUDIN REGULATION OF TIGHT-JUNCTIONAL RESISTANCE IN LOW-RESISTANCE EPITHELIA

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REVIEW ◇ HYPOTHESIS

ABSTRACT. OCCLUDIN IS A TRANSMEMBRANE tight junctional protein whose importance in gating the intercellular space was recently documented in the low-resistance cultured human vaginal and cervical epithelia. Decreases in tight-junctional resistance (R_{TJ}) were associated with occludin degradation, compatible with disassembly of the tight junctions. However, acute and reversible changes in R_{TJ} could also be induced through modulation of assembled tight junctions. Thus, lowering extracellular Ca^{2+} (Ca_0) induced an acute and reversible decrease in R_{TJ}. The present paper presents a novel hypothesis to explain Ca_0 modulation of the R_{TJ}. Accordingly, the functional components of occludin that gate the intercellular space are occludin extracellular loops. The amino acid sequence of human occludin predicts a unique composition of the extracellular loops. Tethered within the proximal parts of the loops are sequences resembling EF-hand – like Ca^{2+}-binding sites. In contrast the distal parts of the loops are protein stretches enriched with hydrophobic amino acids including glycine-rich sequences that are typical to proteins that can readily fold. Also, occludin first and third transmembrane domains from which the loops stem predict α-helices that could anchor the EF-hands into the plasma membrane, and orient the Ca^{2+}-binding domains outwards into the extracellular space. The hypothesis predicts that binding of Ca^{2+} to the EF-hand – like domains will induce inward folding of the EF-hands, along with envelopment of the Ca^{2+}-pockets by the hydrophobic distal parts of the loops. Exteriorization of hydrophobic domains of occludin extracellular loops will lead to formation of hydrophobic bridges between neighboring cells that effectively occlude the intercellular space.

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1. INTRODUCTION

Tight junctions are specialized type of intercellular connections whose main biological role is to restrict the free movement (transport) of water and solutes through the epithelial intercellular (paracellular) route. In addition to controlling transepithelial transport, tight junctions also control lateral migration and sorting of plasma membrane proteins [1], and play a role in regulation of development [2-4] and aging [5,6], water and solutes metabolism [7], and control of inflammation [8-12] infections [13-15] vascular disease [16] organ failure [17,18] and cancer development [19]. Despite their important biological roles, relatively little is known about the mechanisms of operation and regulation of tight junctions. The present paper presents a novel model of tight-junction regulation, based in part on recent discoveries of hormone regulation of tight junctional resistance and expression of tight junctional proteins, and on structure analysis of extracellular loops of the tight junctional protein occludin.

2. BACKGROUND

Tight junctions are part of the apical junctional complex [20] and are composed of tight junction proteins that encircle the epithelial cells in a belt-like manner. Tight junction proteins are composed of cytoplasmic and transmembrane proteins [21]. Cytoplasmic tight-junction proteins couple the transmembrane tight-junctional proteins to the cortical actin and mediate signal-dependent modifications of tight junctional resistance (R_{TJ}) [21]. Transmembrane tight-junctional proteins, including the junctional-adherence molecules (JAMs, present mainly in endothelial cells, see ref. [22,23]), claudins [24-29], and occludins [30,31], are thought to represent linear polymers of transmembrane proteins with extracellular stretches. These associate laterally with tight-junction strands in the apposing membrane of adjacent cells to "seal" the intercellular space.

An important contribution to the understanding of tight junction function were studies using cultures of normal human ectocervical-vaginal epithelial cells (hEVEC) on filters. hEVEC is an established experimental system that models the human female lower reproductive tract epithelia [32]. The latter control secretion of fluid into the lumen, which is important for woman's health and human reproduction. The hEVEC system was used by the author [33-75] and by others [76-85] for studies related to the regulation of the R_{TJ}, and to fit experimental results into mathematical models [42,44-46]. hEVEC form a relatively leaky epithelium where the tight junctional resistance (R_{TJ}) contributes only 75–95% to the total paracellular resistance [45], compared to >99% in other, tighter epithelia [86]. This allows to probe the R_{TJ} and to determine accurately even small changes in the resistance. An added advantage of the hEVEC system for studies of R_{TJ} regulation is the fact that hEVEC cells are estrogen responsive, and estrogen status can affect significantly the R_{TJ} [54-62,65]. The results in hEVEC (described below) were confirmed in other types of low-resistance, "leaky" types of epithelia, including CaSki and HT3 [38].

3. ACUTE MODULATION OF THE R_{TJ}

In hEVEC CaSki and HT3 cells, ATP, added at micromolar concentrations (EC_{50} 0f 3–7 μM) triggers activation of $P2X_4$ receptors and stimulates calcium influx, followed by release of diacylglycerol (DAG) and activation of protein kinase-C (PKC) [37,38,40-42,44-47,50-53,63,64]. One of the consequences of DAG-dependent activation of PKC in those cells is acute threonine dephosphorylation of the 65 kDa (full-length) isoform of occludin [74]. Since hEVEC and CaSki cells (like other epithelial cells) do not express the JAMs, and since DAG-dependent activation of PKC had no significant effect on claudins [74], it was suggested that the ATP-induced acute increase in R_{TJ} was mediated by $P2X_4$-related PKC-dependent threonine dephosphorylation of occludin followed by acute conformational changes of occludin that increased gating of the intercellular space [74]. These results refute the previously held opinion that once assembled, the tight junctions form a solid unmodifiable fence-like structure. Instead, the

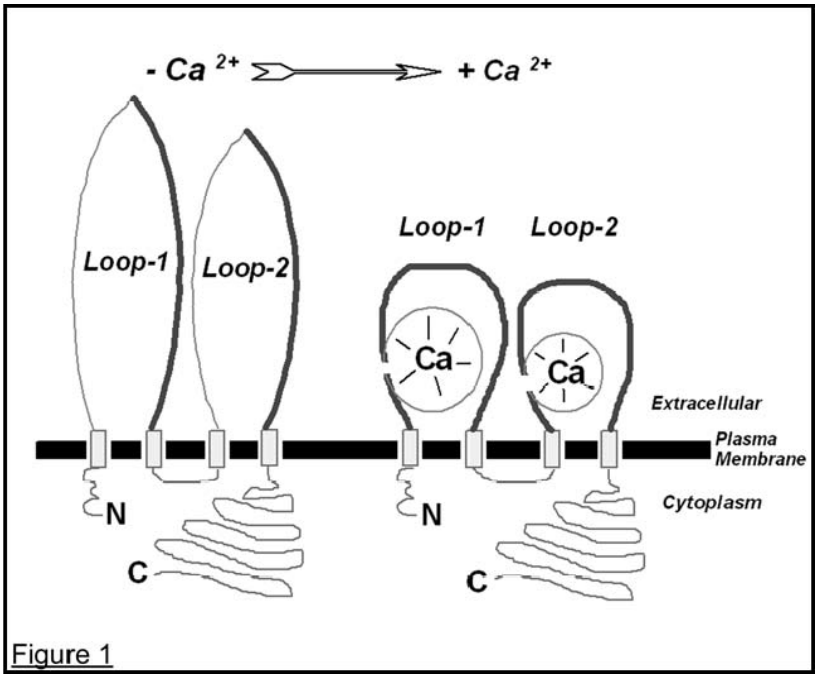

FIGURE 1. PREDICTED STRUCTURE OF OCCLUDIN, AND SCHEMATIC REPRESENTATION OF THE NEW MODEL OF OCCLUDIN REGULATION OF TIGHT-JUNCTION FUNCTION. Occludin is a tetraspan transmembrane protein composed of a short cytoplasmic N-terminus, two extracellular loops (designated Loop 1 and Loop 2), four transmembrane stretches, a short cytoplasmic interconnecting segment, and a long cytoplasmic *C*-terminus. Each of the two extracellular loops can be described in terms of a proximal EF-hand Ca^{2+}-binding domain (narrow lines) and a distal hydrophobic α-helice and "residual-loop segment" (thick lines). In the absence of Ca^{2+} the extracellular loops are maintained in a relaxed state. In the presence of Ca^{2+}, the Ca^{2+}-binding domains transform into calcium-pockets that pack as a core, and cause the hydrophobic distal α-helices and "residualloop segments" to swing outwardly and face the extracellular space. Therefore, each of the occludin extracellular loops can flicker between a hydrophilic surface (no-Ca^{2+}), and a hydrophobic surface (Ca^{2+}-bound). In the Ca^{2+}-bound state, hydrophobic surfaces of extracellular loops of neighboring molecules attach and gate the intercellular space to the free movement of hydrophilic molecules.

results indicate signaling-mediated acute and reversible increase in R_{TJ} of assembled tight junctions.

4. OCCLUDIN AND THE R_{TJ}

Studies in hEVEC and CaSki cells also highlighted the role of occludin in controlling the R_{TJ}. Human occludin type-I is a 65 kDa protein with an apparent 65 amino acid cytosolic N terminus, two extracellular loops of 56 and 48 amino acids separated by an 11 amino acid cytosolic loop; four transmembrane stretches of 11–24 amino acids; and a *C*-terminal tail of 256 amino acids that interacts with the cytoplasmic tight-junction proteins (FIG. 1) [25,31]. Both the *N*- and *C*-terminus domains have phosphorylation sites and the functionally active form of the protein localizing to the tight junction is phosphorylated at serine and threonine residues [21,31] (FIG. 1).

The previously held role of occludin as tight junctional protein became unclear following publications that in some types of cells occludin is not sufficient to form an effective barrier, and other proteins, notably the claudins, are required for optimal occlusion of the paracellular space. For instance, embryonic stem cells lacking occludin maintain a partial permeability barrier [87]; and transfection experiments in mouse fibroblasts have shown that occludin alone forms only partial junctional fibrils [88]. However, many other studies indicated that occludin is a necessary component of the junctional fibrils, and that occludin plays an important role in the gating of tight junctions

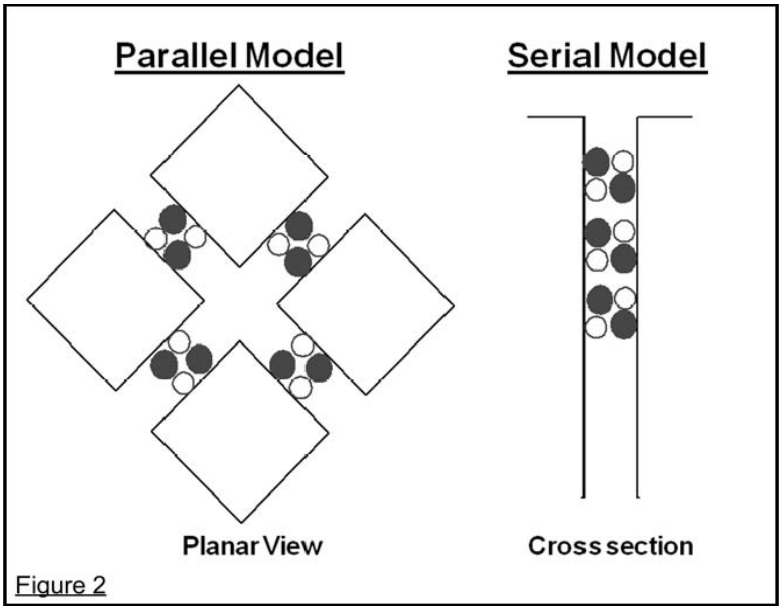

FIGURE 2. SCHEMATIC REPRESENTATION OF THE COOPERATIVE PARALLEL (LEFT) AND SERIAL (RIGHT) MODELS OF TIGHT-JUNCTION ELEMENTS BASED ON Ca^{2+}-DEPENDENT CHANGES IN R_{TJ} IN HEVEC AND CASKI CELLS. Squares are cells; circles are occludin extracellular loops (loop-1 depicted as filled circle, loop-2 as empty circle). The parallel model shows a planar view of four cells sectioned at the level of the tight junctions where each cell attaches to its neighbor via the Ca2+-activated occludin extracellular loops 1 and 2. The parallel model predicts low density of resistive elements that reside at one planar level. Since low density of strands is associated with low R_{TJ}, the cooperative parallel model describes best low resistance epithelia, such as the hEVEC and CaSki. The serial model shows a cross section through two neighboring cells, where the tight junctions are composed of multiple rows of resistive elements that reside serially within the intercellular space. The cooperative serial model describes best high resistance epithelia.

(reviewed in ref. [21]). Thus, depletion of occludin, or transfections of cells with truncated or mutated forms of occludin resulted in aberrant formation of tight junctions, with marked decrease in the number of tight-junction strands and abrogation of the paracellular resistance [89,90]. Other studies showed that transfection of occludin into fibroblasts, which lack endogenous expression of the protein, induces cell-cell adhesiveness [91,92]. Overexpression of occludin in Madin-Darby canine kidney cells resulted in hyperaggregation of globular, hydrophobic, electronmicroscopy-dense particles in regions of the tight junctions, and increased the paracellular resistance [93-95]. Furthermore, treatment of cells with peptides to occludin's extracellular loop 2, but not loop 1, decreased paracellular resistance [95,96] by a mechanism that involved reducing the amount of occludin at the tight junction [96].

In hEVEC and CaSki epithelia, occludin was found to play a significant role in gating the tight junctions. In hEVEC and CaSki cells occludin is present in two main forms: the full-length 65 kDa isoform, and a truncated 50 kDa form [66,74]. Treatment with physiological concentrations of the natural estrogen 17β-estradiol modulated expression of both occludin isoforms: at low concentrations estrogen enhanced synthesis of the 65 kDa isoform, while at higher concentrations, still within the physiological range for the woman, estrogen augmented turnover of the 65 kDa isoform and up-regulation of the 50 kDa isoform. Since estrogen also decreased the R_{TJ}, and because the decreases in R_{TJ} correlated in time with the increase in the 50 kDa occludin form and both effects could be blocked with tamoxifen, it was suggested that estrogen-abrogation of the R_{TJ} involves occludin modulation into the 50 kDa isoform [66].

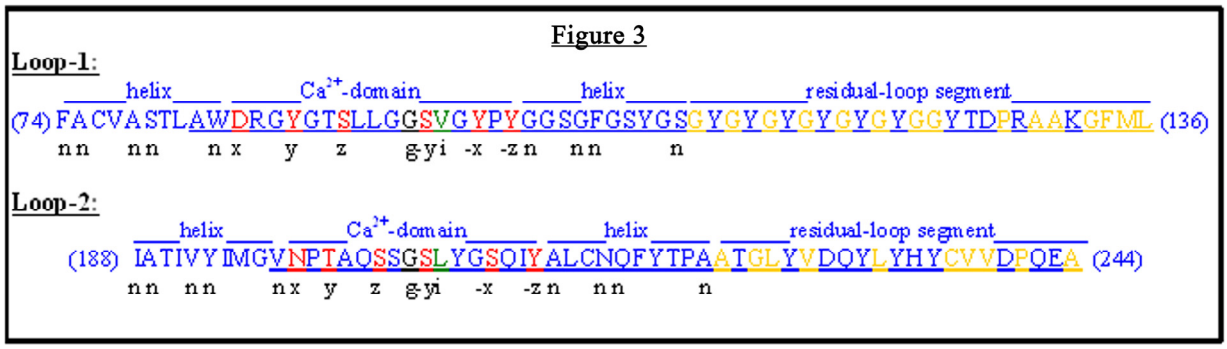

FIGURE 3. PROTEIN STRUCTURE OF HUMAN OCCLUDIN TYPE-1 EXTRACELLULAR LOOPS 1 AND 2 (UNDERLINED) BASED ON THE PREDICTED AMINO ACIDS SEQUENCE OF THE PROTEIN. "n" denotes a hydrophobic amino acid in the α-helix loops, proximal (left) and distal (right) to the Ca2+-binding domains. The amino acids that coordinate binding of the Ca2+ ion are designated x, y, z, -y, -x, and –z, and are shown in red. The glycine (G, shown in black) and the valine (V) and leucine (L) shown in green in the middle of the Ca2+-domain are the "signature" residues (G-x-I/L/V) present in most identified EF-hands.

Moreover, based on the comparative analysis of estrogen effects versus effects of extracellular proteases on occludin expression vis-à-vis changes in R_{TJ} [66] it was recently suggested that the effect of estrogen involves cleavage of occludin near the middle of the extracellular loop 2 [66,74]. This would result in the expression of a defective 50 kDa occludin isoform, and would lead to abrogation of the R_{TJ} by decreasing the availability of intact extracellular loops (FIG. 1). These data can be interpreted in terms of abrogation of the R_{TJ} secondary to disassembly of the tight junctions. Accordingly, estrogen modulation of occludin's extracellular loop 2 leads to abrogation of intercellular occludin-occludin or occludin-claudin connections and results in a decrease in R_{TJ}.

5. EXTRACELLULAR CALCIUM AND THE R_{TJ}

Experiments in hEVEC and CaSki cells also highlighted the role of extracellular calcium (Ca_0) in regulation of the R_{TJ}. We [44,46,51] and others [97-108] have described the dependence of R_{TJ} on Ca^{2+}. Other investigators looked at the effects of calcium on paracellular resistance within hours after lowering extracellular calcium, or after shifting cells back to normal calcium. Studies by others described the "calciumswitch", whereby lowering extracellular calcium abrogates the paracellular resistance, and shifting cells back to normal calcium restores the resistance [97-108]. The studies provided important information about modulation of paracellular resistance secondary to changes in cytosolic calcium, modulation of the cytoskeleton and adherence junctions, affecting the paracellular resistance secondary to changes in cell configuration and intercellular adherence, and metabolic effects of calcium that affect synthesis, folding and insertion of tight-junctional proteins into the plasma membrane [100,102,103,109-111]. However, the results of these studies cannot be explained in terms of direct modulation of tight junctional proteins by calcium, at extracellular domains.

In contrast to studies by others, our published data can be interpreted as direct regulation by calcium of tight-junctional resistance [44,46,51]:

a. Lowering Ca_0 decreased R_{TJ} acutely ($t_{1/2}$ of 2.5 and τ of 4.9 min), and reversibly;

b. The effect was dose-dependent and saturable, with EC_{50}-Ca_0 of 0.3 mM;

c. The effects of Ca_0 on R_{TJ} were not associated with changes in intracellular calcium;

d. Lowering Ca_0 had no effect on occludin mRNA or protein levels, or on cellular distribution of occludin;

e. Within the time-frame of calcium effects on R_{TJ}, lowering Ca_0 had no effect on the tight-junction proteins claudin 4 and ZO-1, or on the

adherence protein E-Cadherin [44,46,51];

f. Among tested bi-tri-valent cations (Ba^{2+}, Cd^{2+}, Co^{2+}, La^{3+}, Mg^{2+}, Mn^{2+}, Sr^{2+}, Zn^{2+}), only Mn^{2+} was found to have partial agonist characteristics of Cao, and it could restore R_{TJ} to near baseline levels [51]. The short time-course of the effects of Ca_0 on R_{TJ} and the reversibility of the effects ruled out modulation of protein synthesis or assembly of tight-junctional proteins as candidates for calcium action [100]. The likely interpretation of our results is that Ca^{2+} interacts with tight-junctional protein(s) directly, at extracellular sites, and induces conformational change that increases gating of the junction.

6. TIGHT-JUNCTION ELEMENTS (UNITS)

Earlier electronmicroscopy freeze-fracture studies showed that the regions of the tight junction which occlude the intercellular space are areas where plasma membranes of neighboring cells come into direct contact. At those sites the tight junctions appear as continuous anastomozing intramembranous strands on the P face, with complementary grooves on the E face [112-120]. The intramembranous particles extend to the extracellular space and form long branching fibrils circumscribing the cell. These areas were functionally interpreted as tight-junction elements (or units). Based on the information presented above, the tight-junction elements are thought to represent linear polymers of transmembrane proteins with extracellular stretches, e.g., occludins and claudins that associate laterally with tight-junction strands in the apposing membrane of adjacent cells to form 'paired' tight-junction strands, and to "seal" the intercellular space. The prevailing theory of gating of the intercellular space is that of homotypic contact across the intercellular spaces between extracellular loops of one type of protein from neighboring adjacent cells, or of heterotypic contact between occludin-claudin extracellular loops [21]. It was suggested that the extracellular loops either form hydrophobic intercellular bridges [21] or isoelectrical domains [121] that gate the intercellular space and effectively block the free movement of water and ions. The specific role of occludin in such a model was supported by studies showing that the addition in the extracellular fluid of peptides analogous to occludin extracellular loops decreased paracellular resistance [96,122,123]. However the role of occludin vis-à-vis the claudins remains unclear.

Tight-junction elements are modeled as pores that flicker between open and closed states [86], such that each small segment can be at one of two states, sealed (rclosed) or open (ropen). According to this model, the net junctional permeability depends on the density of the pores, and on the probability of the open state of the pore. This model predicted that it may be possible to regulate the junctional permeability by modulating the number of the pores, or by modulating the probability of the open state of the pore [46]. The former mode of regulation will most often require protein synthesis and a longer time for expression, while the latter mode of regulation may involve conformational change of existing tight junction proteins, and require seconds to minutes for expression.

The experimental results in the low-resistance hEVEC and CaSki cells have been previously analyzed in terms of functional calcium-regulation of tight-junctional resistance [46]. Accordingly, each tight-junctional element with a microscopic resistance ri contributes to the macroscopic transepithelial resistance such that $RTotal \approx \sum ri$ [46]. This functional model is shown in FIG. 2 and it predicts two Ca^{2+}-binding sites per tight-junction element, where binding of Ca^{2+} in one site is independent of the other. Our previous analyses did not support a non-cooperative bimodal model, which predicts that calcium interacts with junctional elements by binding to single sites, and changes the open/closed probabilities of the junctional elements [46]. Our analyses also did not support a cooperative serial model (FIG. 2, RIGHT PANEL) where the resistive elements reside serially within the intercellular space, and which best describes high resistive epithelia [86]. In contrast, the Ca^{2+}-dependent changes in R_{TJ} in hEVEC and CaSki cells could be best described in terms of a cooperative parallel model (FIG. 2, LEFT PANEL). Accordingly, the macroscopic permeability of the

epithelium is determined by the cooperative opening or closing of the complete set of cell-associated resistive elements where these sets are arranged in parallel. FIG. 2 (LEFT PANEL) describes such a model: it shows a planar view of 4 cells sectioned at the level of the tight junctions, where each cell attaches to its neighbor via two Ca^{2+}-activated occludin extracellular loops. The parallel model predicts low density of resistive elements that reside at one planar level. Since low density of strands is associated with low R_{TJ} [86], the cooperative parallel model describes best low-resistance epithelia, such as hEVEC and CaSki.

At present it is generally accepted that the tight-junction "elements", identified morpho-logically as "strands" and functionally as intercellular resistive elements, are extracellular loops of transmembrane tight junctional proteins [21,121]. Therefore, one mechanism of R_{TJ} control could be the regulation of assembly/disassembly of the junctions, by determining changes in the density of the tight-junction elements (pores). This mechanism has been well described [21,121]. An additional, previously less well understood mechanism of R_{TJ} control was recently suggested by our results [44,46,47,51,66], whereby extracellular loops of assembled transmembrane tight junctional proteins undergo conformational change and flicker between an open, low resistance high permeability state, and a closed, high resistance low permeability state. This mechanism predicts R_{TJ} regulation of assembled tight junctions unrelated to changes in the density of extracellular loops of transmembrane tight junctional proteins between neighboring cells.

At present, little is known about the molecular mechanism by which extracellular loops of transmembrane tight junctional proteins of assembled tight junctions "seal" the intercellular space. The model below presents our novel hypothesis that combines the functional morphologic and biochemical data into a composite mechanism of action. The model focuses on the role of occludins, which based on our previous studies appear to be involved with regulation of the R_{TJ} of assembled tight junctions. This is in contrast to claudins which appear to be involved with regulation of the R_{TJ} through the assembly of tight junctions. Ca_0 effects in low-resistance and high-resistance epithelia

The EC_{50}-Cao levels for changes in R_{TJ} in the low-resistance hEVEC and CaSki cells were about 300 μM [46] compared to 50–100 μM in high-resistance epithelia [100,103]. Three main explanations were offered for this discrepancy:

a. In high-resistance epithelia Ca_0 modulation of R_{TJ} is mediated through changes in cytosolic calcium, which are sensitive to even small changes in Ca_0;

b. The direct calcium-related effects on the open/closed probability of the tight-junctional elements may depend on the density of the junctional elements (*n*) and/or the number of calcium binding sites (*m*). Epithelia with a higher density (higher *n*) have a higher resistance than epithelia with fewer tight-junctional elements [86]. The experimental data in hEVEC and CaSki cells yielded Hill coefficient m of about 2. These data suggest that calcium binds to 2 sites at each functional unit of the tight junctions, and that binding at one site affects binding of calcium at the other site. The analysis also predicts that in a given epithelium the magnitude of m should correlate with the degree of transepithelial resistance, and that the concentration of calcium required to modulate junctional resistance will correlate reciprocally with m, due to the cooperativity of the effect. The latter prediction is supported by the low m (~2) and low RTE (10–50 $\Omega \times cm^2$) across hEVEC and CaSki cultures [46], versus a high m (4-10), and a high RTE (6,000 $\Omega \times cm^2$) in high-resistance epithelia such as cultured A6 cells [103];

c. According to the cooperative model [46], increasing n and/or m may decrease the experimental (macro) EC_{50}-Ca_0 even if the binding affinity of calcium at the molecular level is high. Lowering Ca_0 may not be an effective means to alter the intercellular calcium milieu in tight epithelia. It is therefore possible that the low extracellular calcium required to cause changes in electrical resistance across tight epithelia [100,103] does not reflect the actual calcium concentration in the intercellular space.

7. NEW MODEL: OCCLUDIN REGULATION OF TIGHT-JUNCTION FUNCTION

The author's new hypothesis of occludin regulation of tight-junction function builds on findings in hEVEC and CaSki cells that extracellular loops of the full-length 65 kDa occludin are required for maximal gating of the intercellular space [66,74], and that in these epithelia Cao directly controls the R_{TJ} [44,46,51].

Calcium-binding proteins are composed of one to four repeats of homologous segments, termed "EF hands" (for the Ca^{2+}-binding site between the E and F helices of parvalbumin, [124]). Each EF-hand consists of a α-helix, a Ca^{2+}-binding loop, and a second α-helix [125,126]. Upon activation by Ca^{2+}, the Ca^{2+}-bound – Ca^{2+}-binding protein attaches to target proteins and modulates their activity. The binding affinity of Ca^{2+} to the Ca^{2+}-binding protein is about 10 μM [127]. The binding affinities of activated Ca^{2+}-binding proteins to target proteins varies between 1 nM to 500 μM [128], depending on the structural properties of the interaction, and on the surface shared between the two proteins. Binding of calcium may require a full complement of four EF-hands per Ca^{2+}-binding protein [125], but it can also be effectively accomplished with one or two EF-hands [124,126,128-131]. When not bound to Ca^{2+}, EF-hands form structures with a hydrophobic core and a hydrophilic surface [Rev. in 126,128]. Binding of Ca^{2+} induces conformational change [132] so that the hydrophobic residues swing out to form globular structures with a core containing the Ca^{2+}-pocket, and a hydrophobic shell [126]. The direct approximation of the hydrophobic surface of the Ca^{2+}-binding protein with the hydrophobic surface of the target protein leads to binding and/or activation of the target protein (reviewed in ref. [126,128]). In the Ca^{2+}-binding loop one calcium cation binds to six negatively charged amino acids containing either carbonyl oxygen (aspartate, aspargine, glutamate or glutamine), or negatively charged hydroxyl (serine, threonine or tyrosine). The negatively charged amino acids are spaced by 1 to 3 amino acids, and binding of calcium draws radially the amino acids to form a "calciumpocket" [125,126,128].

The author proposes that extracellular loops of occludin form EF-hand – like Ca^{2+}-binding sites. Based on the published predicted amino acids sequence of human occludin [31], the author has identified amino acid sequences tethered in occludin extracellular loops-1 and -2, that resemble typical EF-hands similar to those previously described in other Ca^{2+}-binding proteins [125,126,128] (FIG. 3). The underlined amino acids in FIG. 3 show the sequences of occludin extracellular loops 1 and 2. Amino-acids numbers in the cartoon are based on published sequence of type-I Human Occludin [31]. The consensus EF-hands are identified by the Tufty-Kretsinger scoring method [133] as indicated below the alignment, where "n" denotes a hydrophobic amino acid in the α-helix loops, proximal (left) and distal (right) to the Ca^{2+}-binding domains. The amino acids that coordinate binding of the Ca^{2+} ion are designated x, y, z, -y, -x, and -z, and are shown in red. The glycine (G, shown in black) and the valine (V) and leucine (L) shown in green in the middle of the Ca^{2+}-domain are the "signature" residues (G-x-I/L/V) present in >95% of identified EF-hands [134,135]. The proposed Ca^{2+}-binding domains in occludin extracellular loops fulfill the criteria of a core of G-x-I/L/V flanked by six negatively charged amino acids containing carbonyl oxygens or negatively charged hydroxyl groups that can bind calcium [125]. Members of the EF-hand superfamily of proteins can display significant variability in their molecular structure [128,136-138], but the core of G-x-I/L/V flanked by six negatively charged amino acids, as predicted in occludin extracellular loops (FIG. 3), is required for binding of Ca^{2+} [128]. The six negatively charged amino acids are spaced by 1 to 3 amino acids, and therefore can form "calcium-pockets" [125]. The proposed α-helix loops fulfill the criteria of amino acids composition, length (8–12 amino acids), proximity, and hydrophobicity [128]. The EF-hand can be flanked proximally by glutamate (E), or any hydrophobic amino acid [126,128].

Based on the analysis in FIG. 3, the author proposes that occludin is a Ca^{2+}-binding protein containing an EF-hand in each of the extracellular

loops. In the absence of Ca^{2+} the loops are maintained in a relaxed (dormant) state (FIG. 1, LEFT PANEL). In the presence of Ca^{2+}, the Ca^{2+}-binding domains transform into calcium-pockets that pack as a core (FIG. 1, right panel), and cause the dormant hydrophobic distal α-helices and "residual-loop segments" to swing outwardly and face the extracellular space (FIG. 1). This allows extracellular loops of occludin projecting off adjacent neighboring cells to interact and form hydrophobic intercellular bridges that effectively seal the intercellular space to the free movement of water and solutes. Consequently, each of occludin extracellular loops can flicker between a hydrophilic surface (no-Ca^{2+}), and a hydrophobic surface (Ca^{2+}-bound).

The above novel hypothesis is supported by the finding that overexpression of occludin results in the formation of hydrophobic fibro-globular structures at extracellular regions of the tight junctions [93-95]. This formation will allow approximation of occludin extracellular loops of adjacent cells [139], and an increase in cell-cell adhesiveness [140,141]. It will subsequently impede the free movement of water, water-soluble molecules and ions in the intercellular space, and thereby result in an increase in the R_{TJ}.

The data in FIG. 3 show a striking similarity in the predicted structure of the EF-hands in the first and second extracellular loops of occludin: occludin first and third transmembrane domains from which the loops stem can be described in terms of α-helices, thereby anchoring the more distal EF-hands into the plasma membrane, and orienting the Ca^{2+}-binding domains outwards into the extracellular space. The Ca^{2+}-binding domains in both loops are of similar lengths (17 and 16 amino acids, respectively), and are continuous with distal α-helices and "residual-loop segments". FIG. 3 also shows that the "residual-loop segments" of the distal halves of the loops are protein stretches enriched with hydrophobic amino acids. Moreover, the "residual-loop segment" of loop-1 contains the unusual glycine-rich sequence (GY)n that is typical to proteins that can readily fold. This predicted structure supports the proposed model of conformational change of the loop following binding of Ca^{2+}.

In summary, based on the above considerations the author proposes that in low-resistance epithelia occludin extracellular loops are the functional domain of the tight junctions that controls gating of the intercellular space. The molecular mechanism of occludin action involves binding of Ca^{2+} to an EF-hand – like domain composing the proximal part of the loops; this induces inward folding of the EF-hand along with envelopment of the Ca^{2+}-pocket by a hydrophobic distal part of the loop. Exteriorization of hydrophobic domains of occludin extracellular loops will lead to formation of hydrophobic bridges between neighboring cells that effectively occlude the intercellular space. Discussion and Conclusions

The new occludin hypothesis raises three questions that need further discussion. The first relates to Ca_0 and its physiological role as regulator of occludin function. The data in low-resistance epithelia showed relatively high EC_{50}-Ca_0 levels for changes in R_{TJ}, 300 μM [46]. This can be explained by correlating the binding affinity of [Ca^{2+}-binding proteins to target proteins] with the [EF-hands conformation status]: a low number of Ca^{2+}-binding sites indicates a small surface shared between the Ca^{2+}-binding protein and the target protein, and would require higher Ca^{2+} concentrations to activate the target protein [128]. Since only two Ca^{2+}-binding sites per occludin are predicted in those cells, calcium-affinity for modulation of R_{TJ} is expected to be low.

This argument raises a second question, whether Cao can reach the mid-micromolar range in view of the tight regulation of extracellular Ca^{2+} at the 1.2–1.5 millimolar range. The explanation is that while plasma mean Ca_{2+} levels are relatively stable, extracellular Ca^{2+} levels at the cell surface can vary due to the presence of cell surface or cell secreted Ca^{2+}-binding proteins [142,143]. Therefore, the experimental EC_{50}-Ca_0 levels for changes in R_{TJ} in hEVEC and CaSki cells are in the predicted physiological Ca_0 levels at the cell surface.

The third and most intriguing question relates to the role of occludin *in vivo*. As discussed above,

the specific role of occludin is debated among different investigators mainly because transfection assays and experiments in occludin-deficient transgenic mice showed that occludin, in contrast to the claudins, is not necessary for gating the intercellular space [121]. Our data in the low-resistance hEVEC and CaSki epithelia and the above described novel model of occludin function suggest a specific role for occludin. Changes in occludin phosphorylation status and expression correlated with changes in R_{TJ} [66,74]. Occludin extracellular loops, in contrast to those of the claudins, contain EF-hands – like domains and may function as Ca^{2+}-binding sites and undergo conformational change in response to changes in Ca_0. In contrast, the structure of claudins extracellular loops predicts a rigid structure that is not compatible with Ca^{2+}-binding characteristics, and claudins gating of the extracellular space appears to depend more on electrostatic forces generated by extracellular loops of neighboring cells than be influenced by Ca_0. Moreover, claudins first extracellular loop is three times longer than the second [24], in contrast to similar lengths of occludin extracellular loops (FIG. 3).

Based on these data, it appears that occludin and the claudins have different biological roles. Claudins are the main building units of the tight junction, and confer the baseline gating properties of the intercellular space. Claudins regulation of the R_{TJ} depends mainly on the density of the proteins: Cells expressing greater density of claudins will tend to form serial-like tight-junctional structures (FIG. 2) and to generate high-resistance epithelia. Since claudins extracellular loops do not tether Ca^{2+}-sensitive mechanisms, R_{TJ} in epithelia containing tight junctions composed of claudins (*i.e.*, high-resistance epithelia) would be less sensitive to Cao regulation. In those cells Ca_0 changes in assembly/disassembly of the tight junctions will depend mainly on Cao-modulation of cytosolic Ca^{2+}. Similar to the claudins, occludin regulation of the R_{TJ} may also depend on its cellular density and expression. Thus, in estrogen-responsive cells estrogen-induced transformation of the full-length 65 kDa form to the truncated 50 kDa form decreases the R_{TJ} [66,74]. However, in contrast to the claudins, occludin gating of the intercellular space can be acutely and reversibly regulated irrespective of its density and expression by at least two known mechanisms, threonine dephosphorylation of occludin intracellular domains (*N*- or *C*-termini) [74], and Ca^{2+} binding to occludin extracellular loops (ref. [46], FIG. 3). Therefore, the combined expression of claudins and occludin provides tight junctions with stability (claudins and occludin) and regulability (occludin). The importance of occludin is apparent in low-resistance epithelia while in high-resistance epithelia the role of occludin is probably masked by the predominance of claudins.

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REFERENCES

[1] SHIN K, FOGG VC AND MARGOLIS B [2006] Tight junctions and cell polarity. Annu Rev Cell Dev Biol [June 13, 2006; Epub ahead of print].

[2] SUN EL AND GONDOS B [1986] Formation of the blood-testis barrier in the rabbit. Cell Tissue Res 243: 575-578.

[3] FLEMING TP, PAPENBROCK T, FESENKO I, HAUSEN P AND SHETH B [2000] Assembly of tight junctions during early vertebrate development. Semin Cell Dev Biol 11: 291-299.

[4] FLEMING TP, SHETH B AND FESENKO I [2001] Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis. Front Biosci 6: D1000-D1007.

[5] MOORADIAN AD [1988] Effect of aging on the blood-brain barrier. Neurobiol Aging 9: 31-39.

[6] LEE EY, LEE SY, LEE TS, CHI JG, CHOI W AND SUH YH [2000] Ultrastructural changes in microvessel with age in the hippocampus of senescence-accelerated mouse (SAM)-P/10. Exp Aging Res 26: 3-14.

[7] MADARA JL, PARKOS C, COLGAN S, NUSRAT A, ATISOOK K AND KAUTZANI P [1992] The movement of solutes and cells across tight junctions. Ann NY Acad Sci 664: 47-60.

[8] HOLLANDER D [1999] Intestinal permeability, leaky gut, and intestinal disorders. Curr Gastroenterol Rep 1: 410-416.

[9] SCHURMANN G, BRUWER M, KLOTZ A, SCHMID KW, SENNINGER N AND ZIMMER KP [1999] Transepithelial transport processes at the intestinal mucosa in inflammatory bowel disease. Int J Colorectal Dis 14: 41-46.

[10] SCHMITZ H, BARMEYER C, FROMM M, RUNKEL N, FOSS HD, BENTZEL CJ, RIECKEN EO AND SCHULZKE JD [1999] Altered tight junction structure contributes to the impaired epithelial barrier function in ulcerative colitis. Gastroenterology 116: 301-309.

[11] JOHNSTON SD, SMYE M AND WATSON RP [2001] Intestinal permeability tests in coeliac disease. Clin Lab 47: 143-150.

[12] YOSHIDA Y, MORITA K, MIZOGUCHI A, IDE C AND MIYACHI Y [2001] Altered expression of occludin and tight junction formation in psoriasis. Arch Dermatol Res 293: 239-244.

[13] SPITZ J, YUHAN R, KOUTSOURI A, BLATT C, ALVERDY J AND HECHT G [1995] Enteropathogenic Eschrichia coli adherence to intestinal epithelial monolayers diminished barrier function. Am J Physiol 268: G374-G379.

[14] HECHT G, POTHOULAKIS C, LAMONT JT AND MADARA JL [1988] Clostridium difficile toxin a perturbs cytoskeletal structure and tight junction permeability of cultured human intestinal epithelial monolayers. J Clin Invest 82: 1516-1524.

[15] TAFAZOLI F, ZENG CQ, ESTES MK, MAGNUSSON KE AND SVENSSON L [2001] NSP4 Enterotoxin of Rotavirus induces paracellular leakage in polarized epithelial cells. J Virology 75: 1540-1546.

[16] LEE WC, CHAO WT AND YANG VC [2001] Effects of high-cholesterol diet on the interendothelial clefts and the associated junctional complexes in rat aorta. Atherosclerosis 155: 307-312.

[17] MEIER-ABT PJ [1990] Cellular mechanisms of intrahepatic cholestasis. Drugs 40(suppl 3): 84-97.

[18] TAKAKI Y, HIRAI S, MANABE N, IZUMI Y, HIROSE T, NAKAYA M, SUZUKI A, MIZUNO K, AKIMOTO K, TSUKITA S, SHUIN T AND OHNO S [2001] Dynamic changes in protein components of the tight junction during liver regeneration. Cell Tissue Res 305: 399-409.

[19] POLAK-CHARCON S, SHOHAM J AND BEN-SHAUL Y [1980] Tight junctions in epithelial cells of human fetal hindgut, normal colon, and colon adenocarcinoma. J Natl Cancer Inst 65: 53-62.

[20] BISSELL MN, AND NELSON WJ [1999] Cell-to-cell contact and extracellular matrix. Curr Opin Cell Biol 11: 11-22.

[21] TSUKITA S, FURUSE M AND ITOH M [2001] Multifunctional strands in tight junctions. Nature Rev Mol Cell Biol 2: 285-293.

[22] MARTIN-PADURA I, LOSTAGLIO S, SCHNEEMANN M, WILLIAMS L, ROMANO M, FRUSCELLA P, PANZERI C, STOPPACCIARO A, RUCO L, VILLA A, SIMMONS D AND DEJANA E [1998] Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. J Cell Biol 142: 117-127.

[23] AURRAND-LONS MA, DUNCN L, DU PASQUIRE L AND IMHOF BA [2000] Cloning of JAM2 and JAM3: An emerging junctional adhesion molecular family? Curr Top Microbiol Immunol 251: 91-98.

[24] FURUSE M, FUJITA K, HIIRAGI T, FUJIMOTO K AND TSUKITA S [1998] Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 141: 1539-1550.

[25] FURUSE M, SASAKI H, FUJIMOTO K AND TSUKITA S [1998] A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 143: 391-401.

[26] MORITA K, FURUSE M, FUJIMOTO K AND TSUKITA S [1999] Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96: 511-516.

[27] TSUKITA S AND FURUSE M [2000] Pores in the wall: Claudins constitute tight junction strands containing aqueous pores. J Cell Biol 149: 13-16.

[28] HEISKAL M, PETERSON PA AND YANG Y [2000] The roles of claudin superfamily proteins in paracellular transport. Traffic 2: 92-98.

[29] VAN ITALLIE CM AND ANDERSON JM [2006] Claudins and epithelial paracellular transport. Annu Rev Physiol 68: 403-429.

[30] FURUSE M, HIRASE T, ITOH M, NAGAFUCHI A, YONEMURA S, TSUKITA S AND TSUKITA S [1993] Occludin: A novel integral membrane protein localizing at tight junctions. J Cell Biol 123: 1777-1788.

[31] ANDO-AKATSUKA Y, SAITOU M, HIRASE T, KISHI M, SAKAKIBARA A, ITOH M, YONEMURA S, FURUSE M AND TSUKITA S [1996] Interspecies diversity of the occludin sequence: cDNA cloning of human, mouse, dog and ratkangaroo homologues. J Cell Biol 133: 43-47.

[32] GORODESKI GI, ROMERO MF, HOPFER U, RORKE E, UTIAN WH AND ECKERT RL [1994] Human uterine cervical epithelial cells grown on permeable support—A new model for the study of differentiation and transepithelial transport. Differentiation 56: 107-118.

[33] GORODESKI GI, ECKERT RL, UTIAN WH, SHEEAN L AND RORKE EA [1989] Retinoids, sex steroids and glucocorticoids regulate ectocervical cell envelope formation but not the level of the envelope precursor, involucrin. Differentiation 42: 75-80.

[34] GORODESKI GI, ECKERT RL, UTIAN WH AND RORKE EA

[1990] Maintenance of *in vivo*-like keratin expression, sex steroid responsiveness and estrogen receptor expression in cultured human ectocervical epithelial cells. Endocrinology 126: 399-406.

[35] GORODESKI GI, ECKERT RL, UTIAN WH, SHEEAN L AND RORKE EA [1990] Cultured human ectocervical epithelial cell differentiation is regulated by the combined direct actions of sex steroids, glucocorticoids and retinoids. J Clin Endocrinol Metab 70: 1624-1630.

[36] SIZEMORE N, KASTURI L, GORODESKI G, ECKERT RL, JETTEN AM AND RORKE EA [1993] Retinoid regulation of human ectocervical epithelial cell transglutaminase activity and keratin gene expression. Differentiation 54: 219-225.

[37] GORODESKI GI, HOPFER U, ECKERT RL, UTIAN WH, DE-SANTIS BJ, RORKE ER AND ROMERO MF [1994] ATP decreases acutely and reversibly transport through the paracellular pathway in human uterine cervical cells. Am J Physiol 266: C1692-C1698.

[38] GORODESKI GI, MERLIN D, DE SANTIS BJ, FRIEDEN KA, HOPFER U, ECKERT RL AND ROMERO MF [1994] Characterization of paracellular permeability in cultured human cervical epithelium: Regulation by extracellular ATP. J Soc Gynecol Invest 1: 225-233.

[39] JACOBBERGER JW, SIZEMORE N, GORODESKI GI AND RORKE EA [1995] Transforming growth factor β regulation of epidermal growth factor receptor in ectocervical epithelial cells. Exp Cell Res 220: 390-396.

[40] GORODESKI GI, HOPFER U, DE SANTIS BJ, ECKERT RL, RORKE ER AND UTIAN WH [1995] Biphasic regulation of paracellular permeability in human cervical cells by two distinct nucleotide receptors. Am J Physiol 268: C1215-C1226.

[41] GORODESKI GI AND HOPFER U [1995] Regulation of the paracellular permeability of cultured human cervical epithelium by a purinergic P2-receptor. J Soc Gynecol Invest 2: 716-720.

[42] GORODESKI GI, DE SANTIS BJ, GOLDFARB J, UTIAN WH AND HOPFER H [1995] Osmolar changes regulate the paracellular permeability of cultured human cervical epithelium. Am J Physiol 269: C870-C877.

[43] MACINGA D, JAIN V, SIZEMORE N, GORODESKI GI, ECKERT RL AND RORKE EA [1995] Tamoxifen regulation of ectocervical cell differentiation. J Soc Gynecol Investig 2: 754-761.

[44] GORODESKI GI, PETERSON D, DE SANTIS BJ AND HOPFER U [1996] Nucleotide-receptor mediated decrease of tight-junctional permeability in cultured human cervical epithelium. Am J Physiol 270: C1715-C1725.

[45] GORODESKI GI [1996] The cultured human cervical epithelium: A new model for studying transepithelial paracellular transport. J Soc Gynecol Invest 3: 267-280.

[46] GORODESKI GI, WENWU J AND HOPFER U [1997] Extracellular Ca^{2+} directly regulates tight junctional permeability in the human cervical cell line CaSki. Am J Physiol 272: C511-C524.

[47] GORODESKI GI AND GOLDFARB J [1997] Extracellular ATP regulates transcervical permeability by modulating two distinct paracellular pathways. Am J Physiol 272: C1602-C1610.

[48] GORODESKI GI, ECKERT RL, PAL D, UTIAN WH AND RORKE EA [1997] Retinoids regulate tight junctional resistance of cultured human cervical cells. Am J Physiol 273: C1707-C1713.

[49] GORODESKI GI AND GOLDFARB J [1998] Seminal fluid factor increases the resistance of the tight junctional complex of the cultured human cervical epithelium CaSki. Fertil Steril 69: 309-317.

[50] GORODESKI GI, PAL D, RORKE EA, ECKERT RL AND BURFEIND P [1998] Retinoids modulate P2U purinergic receptor-mediated changes in transcervical paracellular permeability. Am J Physiol 274: C1108-C1116.

[51] GORODESKI GI, HOPFER U AND WENWU J [1998] Purinergic receptor induced changes in paracellular resistance across cultures of human cervical cells are mediated by two distinct cytosolic calcium related mechanisms. Cell Biochem Biophys 29: 281-306.

[52] GORODESKI GI AND WHITTEMBURY J [1998] A novel fluorescence chamber for the determination of volume changes in human CaSki cell cultures attached on filters. Cell Biochem Biophys 29: 307-332.

[53] GORODESKI GI, BURFEIND F, UIN GAN S, PAL D AND ABDUL-KARIM F [1998] Regulation by retinoids of P2Y2 nucleotide receptor mRNA in human uterine cervical cells. Am J Physiol 275: C758-C765.

[54] GORODESKI GI [1998] Estrogen increases the permeability of the cultured human cervical epithelium by modulating cell deformability. Am J Physiol 275: C888-C899.

[55] GORODESKI GI AND PAL D [2000] Involvement of estrogen receptors α and β in the regulation of cervical permeability. Am J Physiol 278: C689-C696.

[56] GORODESKI GI [2000] Role of nitric oxide and cGMP in the estrogen regulation of cervical epithelial permeability. Endocrinology 141: 1658-1666.

[57] GORODESKI GI [2000] Effects of menopause and estrogen on cervical epithelial permeability. J Clin Endocrinol Metab 85: 2584-2595.

[58] GORODESKI GI [2000] NO increases permeability of cultured human cervical epithelia by cGMP-mediated increase in G-actin. Am J Physiol 278: C942-C952.

[59] GORODESKI GI [2000] Calcium regulates estrogen increase in permeability of cultured CaSki epithelium by eNOS dependent mechanism. Am J Physiol 279: C1495-C1505.

[60] GORODESKI GI [2000] cGMP-dependent ADP-depolymerization of actin mediates estrogen increase in human cervical epithelia permeability. Am J Physiol 279: C2028-C2036.

[61] GORODESKI GI [2000] Vaginal-cervical epithelial permeability decreases after menopause. Fertil Steril 76: 753-761.

[62] GORODESKI GI [2001] Estrogen biphasic regulation of paracellular permeability of cultured human vaginal-cervical epithelia. J Clin Endocrinol Metab 86: 4233-4243.

[63] GORODESKI GI [2002] Regulation of transcervical permeability by two distinct P2purinergic receptor mechanisms. Am J Physiol 282: C75-C83.

[64] GORODESKI GI [2002] Expression regulation and function of $P2X_4$ receptor in human cervical epithelial cells. Am J Physiol 282: C84-C93.

[65] GORODESKI GI [2004] Estrogen attenuates $P2X_7$-R-mediated apoptosis of uterine cervical cells by blocking calcium influx. Nucleosides Nucleic Acids 23: 209-215.

[66] ZENG R, LI X AND GORODESKI GI [2004] Estrogen abrogates transcervical tight junctional resistance by acceleration of Occludin modulation. J Clin Endocrinol Metab 89: 5145-5155.

[67] WANG Q, WANG L, FENG YH, LI X, ZENG R AND GORODESKI GI [2004] $P2X_7$-receptor mediated apoptosis of human cervical epithelial cells. Am J Physiol 287: C1349-C1358.

[68] WANG Q, LI X, WANG L, FENG YH, ZENG R AND GORODESKI GI [2004] Anti-apoptotic effects of estrogen in normal and in cancer human cervical epithelial cells. Endocrinology 145: 5568-5579.

[69] GORODESKI GI [2005] Aging and estrogen effects on transcervical-transvaginal epithelial permeability. J Clin Endocrinol Metab 90: 345-351.

[70] WANG L, FENG YH AND GORODESKI GI [2005] EGF facilitates epinephrine inhibition of $P2X_7$-receptor mediated pore formation and apoptosis: A novel signaling network. Endocrinology 146: 164-174.

[71] GORODESKI GI, HOPFER U, LIU CC AND MARGLES E [2005] Estrogen acidifies vaginal pH by upregulation of proton secretion via the apical membrane of vaginal-ectocervical epithelial cells. Endocrinology 146: 816-824.

[72] FENG YH, WANG L, WANG Q, LI X, ZENG R AND GORODESKI GI [2005] ATP ligation stimulates GRK-3-mediated phosphorylation and β-arrestin-2 and dynamin-dependent internalization of the $P2X_7$-receptor. Am J Physiol 288: C1342-C1356.

[73] GORODESKI GI [2005] Effects of estrogen on proton secretion via the apical membrane in vaginal-ectocervical epithelial cells of postmenopausal women. Menopause 12: 679-684.

[74] ZHU L, LI X, ZENG R AND GORODESKI GI [2006] Changes in tight junctional resistance of the cervical epithelium are associated with modulation of content and phosphorylation of occludin 65 kDa and 50 kDa forms. Endocrinology 147: 977-989.

[75] FENG YH, LI X, WANG L, ZHOU L AND GORODESKI GI [2006] A truncated $P2X_7$ receptor variant ($P2X_7$-j) endogenously expressed in cervical cancer cells antagonizes the full-length $P2X_7$ receptor through hetero-oligomerization. J Biol Chem 281: 17228-17237.

[76] KASTURI L, SIZEMORE N, ECKERT RL, MARTIN K AND RORKE EA [1993] Calcium modulates cornified envelope formation, involucrin content, and transglutaminase activity in cultured human ectocervical epithelial cells. Exp Cell Res 205: 84-90.

[77] HEMBREE JR, AGARWAL C, BEARD RL, CHANDRARATNA RA AND ECKERT R [1996] Retinoid X receptor-specific retinoids inhibit the ability of retinoic acid receptor-specific retinoids to increase the level of insulin-like growth factor binding protein3 in human ectocervical epithelial cells. Cancer Res 56: 1794-1799.

[78] AGARWAL C, CHANDRARATNA RA, JOHNSON AT, RORKE EA AND ECKERT RL [1996] AGN193109 is a highly effective antagonist of retinoid action in human ectocervical epithelial cells. J Biol Chem 271: 12209-12212.

[79] AGARWAL C, CHANDRARATNA RA, TENG M, NAGPAL S, RORKE EA AND ECKERT RL [1996] Differential regulation of human ectocervical epithelial cell line proliferation and differentiation by retinoid x receptor- and retinoic acid receptor-specific retinoids. Cell Growth Differentiation 7: 521-530.

[80] HEMBREE JR, LAMBERT A, AGARWAL C, EFIMOVA T AND ECKERT RL [1999] Insulin-like growth factor binding protein-3 does not mediate the interferon-dependent suppression of human ectocervical epithelial cell proliferation. Int J Oncol 14: 1163-1168.

[81] AGARWAL C, RORKE EA, IRWIN JC AND ECKERT RL [1991] Immortalization by human papillomavirus type 16 alters retinoid regulation of human ectocervical epithelial cell differentiation. Cancer Res 51: 3982-3989.

[82] CHOO CK, RORKE EA AND ECKERT RL [1993] Calcium regulates the differentiation of human papillomavirus type 16 (HPV16) immortalized ectocervical epithelial cells, but not the expression of the papillomavirus E6 and E7 oncogenes. Exp Cell Res 208: 161-169.

[83] SIZEMORE N AND RORKE EA [1993] Human papillomavirus 16 immortalization of normal human ectocervical epithelial cells alters retinoic acid regulation of cell growth and epidermal growth factor receptor expression. Cancer Res 53: 4511-4517.

[84] RORKE EA AND JACOBBERGER JW [1995] Transforming growth factor-β1 (TGFβ1) enhances apoptosis in human papillomavirus type 16-immortalized human ectocervical

epithelial cells. Exp Cell Res 216: 65-72.

[85] SIZEMORE N, CHOO CK, ECKERT RL AND RORKE EA [1998] Transcriptional regulation of the EGF receptor promoter by HPV16 and retinoic acid in human ectocervical epithelial cells. Exp Cell Res 244: 349-356.

[86] CEREIJIDO, M [1991] Evolution of ideas on the tight junction. In: CEREIJIDO M (EDITOR), Tight-Junctions. Boca Raton, Ann Arbor, London, CRC Press, pp. 1-14.

[87] SAITOU M, FUJIMOTO K, DOI Y, ITOH M, FUJIMOTO T, FURUSE M, TAKANO H, NODA T AND TSUKITA S [1998] Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J Cell Biol 141: 397-408.

[88] KUBOTA K, FURUSE M, SASAKI H, SONODA N, FUJITA K, NAGAFUCHI A AND TSUKITA S [1999] Ca^{2+}-independent cell-adhesion activity of claudins, a family of integral membrane proteins localized at tight junctions. Curr Biol 9: 1035-1038.

[89] BALDA MS, WHITNEY JA, FLORES C, GONZALEZ-MARISCAL L, CEREIJIDO M AND MATTER K [1996] Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by the expression of a mutant tight junction membrane protein. J Cell Biol 134: 1031-1049.

[90] BALDA MS, FLORES-MALDONADO C, CEREIJIDO M AND MATTER K [2000] Multiple domains of occludin are involved in the regulation of paracellular permeability. J Cell Biochem 78: 85-96.

[91] HUBER D, BALDA MS AND MATTER K [2000] Occludin modulates transepithelial migration of neutrophils. J Biol Chem 275: 5773-5778.

[92] SAITOU M, ANDO-AKATSUKA Y, ITOH M, FURUSE M, INAZAWA J, FUJIMOTO K AND TSUKITA, S [1997] Mammalian occludin in epithelial cells: Is expression and subcellular distribution. Eur J Cell Biol. 73: 22-231.

[9] FURUSE M, FUJIMOTO K, SATO N, HIRASE T, TSUKITA S AND TSUKITA S [1996] Overexpression of occludin, a tight junction-associated integral membrane protein, induces the formation of intracellular multilamellar bodies bearing tight junction-like structures. J Cell Sci 109: 429-435.

[94] MCCARTHY KM, SKARE IB, STANKEWICH MC, FURUSE M, TSUKITA S, ROGERS RA, LYNCH RD AND SCHNEEEBERGER EE [1996] Occludin is a functional component of the tight junction. J Cell Sci 109: 2287-2298.

[95] MEDINA R, RAHNER C, MITIC LL, ANDERSON JM AND VAN ITALLIE CM [2000] Occludin localization at the tight junction requires the second extracellular loop. J Membr Biol 178: 235-247.

[96] WONG V AND GUMBINER BM [1997] A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J Cell Biol 136:399-409.

[97] BALDA MS, GONZALES-MARISCAL L. MACIAS-SILVA M, TORRES-MARQUEZ ME, GARCIA SAINZ JA AND CEREIJIDO M [1991] Assembly and sealing of tight junctions: possible participation of G- proteins, phospholipase C, protein kinase C and calmodulin. J Membr Biol 122: 193-202.

[98] CEREIJIDO M, ROBBINS ES, DOLAN WJ, ROTUNNO CA AND SABATINI DD [1978] Polarized monolayers formed by epithelial cells on a permeable and translucent support. J Cell Biol 77: 853-880.

[99] MARTINEZ-PALOMO A, MEZA I, BEATY G AND CEREIJIDO M [1980] Experimental modulation of occluding junctions in a cultured transporting epithelium. J Cell Biol 87: 736-745.

[100] GONZALEZ-MARISCAL L, CONTRERAS RG, BOLIVAR JJ, PONCE A, CHAVEZ DE RAMIREZ B AND CEREIJIDO M [1990] Role of calcium in tight junction formation between epithelial cells. Am J Physiol 259: C978-C986.

[101] NIGAM SK, RODRIGUEZ-BOULAN E AND SILVER RB [1992] Changes in intracellular calcium during the development of epithelial polarity and junctions. Proc Natl Acad Sci USA 89: 6162-6166.

[102] CONTRERAS RG, MILLER JH, ZAMORA M, GONZALEZ-MARISCAL L AND CEREIJIDO M [1992] Interaction of calcium with the plasma membrane of epithelial (MDCK) cells during junction formation. Am J Physiol 263: C313-C318.

[103] JOVOV B, LEWIS SA, CROWE WE, BERG JR, AND WILLS NK [1994] Role of intracellular Ca^{2+} in modulation of tight junction resistance in A6 cells. Am J Physiol 266: F775-F784.

[104] STUART RO, SUN A, PANICHAS M, HEBERT SC, BRENNER BM, AND NIGAM SK [1994] Critical role for intracellular calcium in tight junction biogenesis. J Cell Physiol. 159: 423-433.

[105] SIGURDSON SL AND LWEBUGA MUKASA JS [1994] Divalent cation dependent regulation of rat alveolar epithelial cell adhesion and spreading. Exp Cell Res 213: 719-727.

[106] STUART RO, SUN A, BUSH KT AND NIGAM SK [1996] Dependence of epithelial intercellular junction biogenesis on thapsigargin-sensitive intracellular calcium stores. J Biol Chem 271: 13636-13641.

[107] YE J, TSUKAMOTO T, SUN A AND NIGAM SK [1999] A role for intracellular calcium in tight junction reassembly after ATP depletion-repletion. Am J Physiol 277: F524-F532.

[108] MA TY, TRAN D, NGUYEN D, MERRYFIELD M AND TARNAWSKI A [2000] Mechanism of extracellular calcium regulation of intestinal epithelial tight junction permeability role of cytoskeletal involvement. Microsc

Res Tech 51: 156-168.

[109] MELDOLESI J, CASTIGLIONI G, PARMA R, NASSIVERA N AND DE-CAMILLI P [1978] Ca^{2+}-dependent disassembly and reassembly of occluding junctions in guinea pig pancreatic acinar cells. J Cell Biol 79: 156-172.

[110] PITELKA DR, TAGGART BN AND HAMAMOTO ST [1983] Effects of extracellular calcium depletion on membrane topography and occluding junctions of mammary epithelial cells in culture. J Cell Biol 96: 613-624.

[111] VOLBERG T, GEIGER B, KARTENBECK J AND FRANKE WW [1986] Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca^{2+} ions. J Cell Biol 102: 1832-1842.

[112] FARQUHAR MG AND PALADE GE [1963] Junctional complexes in various epithelia. J Cell Biol 17: 375-412.

[113] CLAUDE P AND GOODENOUGH DA [1973] Fracture faces of zonulae occludentes from 'tight' and 'leaky' epithelia. J Cell Biol 58: 390-400.

[114] STAEHELIN LA [1973] Further observations on the fine structure of freeze-cleaved tight junctions. J Cell Sci 13: 763-786.

[115] MARTINEZ-PALOMO A AND ERLIJ D [1975] Structure of tight junctions in epithelia with different permeability. Proc Natl Acad Sci USA 72: 4487-4491.

[116] CLAUDE P [1978] Morphological factors influencing transepithelial permeability: a model for the resistance of the zonula occludens. J Membr Biol 39: 219-232.

[117] KACHAR B AND PINTO DA SILVA P [1981] Rapid massive assembly of tight junctions strands. Science 213: 541-544.

[118] MADARA JL AND DHARMSATHAPHORN K [1985] Occluding junction structure function relationships in a cultured epithelial monolayer. J Cell Biol 101: 2124-2133.

[119] MARCIAL MA, CARLSON SL AND MADARA JL [1987] Partitioning of paracellular conductance along the ileal crypt-villus axis: A hypothesis based on structural analysis with detailed consideration of tight junction-structure function relationships. J Membr Biol 80: 59-70.

[120] LANE NJ, REESE TS AND KACHAR B [1992] Structural domains of the tight junctional intramembrane fibrils. Tissue Cell. 24: 291-300.

[121] VAN ITALLIE CM AND ANDERSON JM [2004] The molecular physiology of tight junction pores. Physiology 19: 331-338.

[122] LACAZ-VIEIRA F, JAEGER MMM, FARSHORI P AND KACHAR B [1999] Small synthetic peptides homologous to segments of the first external loop of occludin impair tight junction resealing. J Membr Biol 168: 289-297.

[123] TAVELIN S, HASHIMOTO K, MALKINSON J, LAZOROVA L, TOTH I AND ARTURSSON P [2003] A new principle for tight junction modulation based on occludin peptides. Mol Pharmacol 64: 1530-1540.

[124] KRETSINGER RH [1980] Structure and evolution of calcium-modulated proteins. CRC Crit Rev Biochem 8: 119-174.

[125] KLEE CB, CROUCH TH AND RICHMAN PG [1980] Calmodulin. Ann Rev Biochem 49: 489-515.

[126] JURADO LA, CHOCKALINGAM PS AND JARRETT HW [1999] Apocalmodulin. Physiolo Rev 79: 661-682.

[127] COX JA [1988] Interactive properties of calmodulin. Biochem J 249: 621-629.

[128] YAP KL, AMES JB, SWINDELLS MB AND IKURA M [1999] Diversity of conformational states and changes within the EF-hand protein superfamily. Proteins: Structure, Function and Genetics 37: 499-507.

[129] TREWHELLA J, BLUMENTHAL DK, ROKOP SE AND SEEGER PA [1990] Small-angle scattering studies show distinct conformations of calmodulin in its complexes with two peptides based on the regulatory domain of the catalytic subunit of phosphorylase kinase. Biochemistry 29: 9316-9324.

[130] PETERSON BZ, LEE JS, MULLE JG, WANG Y, DE LEON M AND YUE DT [2000] Critical determinants of Ca^{2+}-dependent inactivation within an EF-hand motif of L-type Ca^{2+} channels. Biophys J 78: 1906-1920.

[131] BRAUN AP AND SY L [2001] Contribution of potential EF hand motifs to the calcium-dependent gating of a mouse brain large conductance, calcium-sensitive K^+ channel. J Physiol 533 (Part 3): 681-695.

[132] ZHANG M, TANAKA T AND IKURA M [1995] Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. Nature Struct Biol 2: 758-767.

[133] TUFTY RM AND KRETSINGER RH [1975] Troponin and parvalbumin calcium binding regions predicted in myosin light chain and T4 lysozyme. Science 187: 167-169.

[134] MARSDEN BJ, SHAW GS AND SYKES BD [1990] Calcium binding proteins. Elucidating the contributions to calcium affinity from an analysis of species variants and peptide fragments. Biochem Cell Biol 68: 587-601.

[135] FALKE JJ, DRAKE SK, HAZARD AL AND PEERSEN OB [1994] Molecular tuning of ion binding to calcium signaling proteins. Quarterly Rev Biophys 27: 219-290.

[136] IKURA M, ALATTIA JR AND ISHIMA R [1997] Structural bases of calcium-dependent cell adhesion and intracellular calcium signaling. Proceedings of the Tenth International Symposium on Calcium-binding Proteins and Calcium Function in Health and Disease, June 17–21, Lund, Sweden.

[137] NELSON MR AND CHAZIN WJ [1998] Structures of EF-hand Ca^{2+}-binding proteins: Diversity in the organization, packing and response to Ca^{2+} binding.

Biometals 11: 297-318.

[138] AMES JB, TANAKA T, STRYER L AND IKURA M [1996] Portrait of a myristoyl switch protein. Curr Opin Struct Biol 6: 432-438.

[139] CHEN Y, LU Q, SCHNEEBERGER EE AND GOODENOUGH DA [2000] Restoration of tight junction structure and barrier function by downregulation of the mitogen-activated protein kinase pathway in Ras-transformed Madin-Darby canine kidney cells. Mol Biol Cell 11: 849-862.

[140] ANDERSON JM AND VAN ITALLIE CM [1995] Tight junctions and the molecular basis for regulation of paracellular permeability. Am J Physiol 269: G467-G475.

[141] VAN ITALLIE CM AND ANDERSON JM [1997] Occludin confers adhesiveness when expressed in fibroblasts. J Cell Sci 110: 1113-1121.

[142] MUNSHI HG, WU YI, ARIZTIA EV AND STACK MS [2002] Calcium regulation of matrix metalloproteinase-mediated migration in oral squamous cell carcinoma cells. J Biol Chem 277: 41480-41488.

[143] HWANG M, KALININ A AND MORASSO MI [2005] The temporal and spatial expression of the novel Ca^{++}-binding proteins, Scarf and Scarf2, during development and epidermal differentiation. Gene Expr Patterns 5:801-808.

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