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Topical Review

Critical Role of Tight Junctions in Drug Delivery across Epithelial and Endothelial Cell Layers

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Abstract. Epithelia in multicellular organisms constitute the frontier that separates the individual from the environment. Epithelia are sites of exchange as well as barriers, for the transit of ions and molecules from and into the organism. Therapeutic agents, in order to reach their target, frequently need to cross epithelial and endothelial sheets. Two routes are available for such purpose: the transcellular and the paracellular pathways. The former is employed by lipophilic drugs and by molecules selectively transported by channels, pumps and carriers present in the plasma membrane. Hydrophilic molecules cannot cross biological membranes, therefore their transepithelial transport could be significantly enhanced if they moved through the paracellular pathway. Transit through this route is regulated by tight junctions (TJs). The discovery in recent years of the molecular mechanisms of the TJ has allowed the design of different procedures to open the paracellular route in a reversible manner. These strategies could be used to enhance drug delivery across epithelial and endothelial barriers. The procedures employed include the use of peptides homologous to external loops of integral TJ proteins, silencing the expression of TJ proteins with antisense oligonucleotides and siRNAs as well as the use of toxins and proteins derived from microorganisms that target TJ proteins.

Key words: Tight junctions — Drug delivery — Paracellular pathway — Claudins — Occludin — ZO-1

Introduction

Epithelia constitute sheets of cells closely bound together, which delimit unique environments in opposing compartments (Fig 1*A*). Epithelial tissues include the epidermis, the surfaces of the eye and those that cover the lumen of the digestive, respiratory, reproductive and urinary tracts, as well as the glandular ducts. The polarized nature of epithelial cells allows them to display barrier, secretory and absorptive functions. Hence the apical plasma membrane facing the lumen exhibits a distinctive morphological appearance characterized by the presence of microvilli and a protein and lipid composition that differs markedly from that found at the basolateral membrane in contact with the tissue parenchyma. Vectorial transport processes across epithelial sheets depend on cell polarity, and can be exemplified with transepithelial Na^+ transport that requires the participation of an amiloride-sensitive Na^+ channel located at the apical membrane, and of a Na^+, K^+-ATPase situated at the basolateral surface (Cereijido, Shoshani & Contreras, 2001).

Due to the fluid nature of biological membranes, proteins and lipid could in principle move laterally within the plasma membrane from one cell surface to another. However, in epithelial cells this is not the case because transmembrane proteins are linked to scaffolding proteins that anchor them to the cytoskeleton, and due to the presence of tight junctions (TJs), which function as a fence that blocks the movement of proteins and lipids within the plasma membrane, between the apical and the basolateral surfaces.

TJs are located at the uppermost portion of the lateral plasma membrane. In contrast to other intercellular junctions like desmosomes and gap junctions, which establish spot welds and pipelike channels at individual points along the lateral membrane, TJs constitute a continuous belt that encircles every epithelial cell and virtually fuses the plasma membranes of neighboring cells. When the interior leaflets of the plasma membrane are analyzed by freeze-fracture electron microscopy, the TJ appears as a network of anastomosing strands in the P face and of corre-

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sponding grooves on the E face, that surround the epithelial cells bellow the microvillus of the apical surface.

TJs have both gate and fence functions. Thus, they work as a semipermeable barrier that controls the passage of ions, water and molecules through the intercellular space, also called the paracellular route (Cereijido, Shoshani & Contreras, 2000) and, as above mentioned, act as a fence that maintains the polarized epithelial phenotype.

Endothelial cells also have TJs. Those from non-neural vessels are somewhat different from those in epithelia (Bazzoni & Dejana, 2004a) since they can be found at the uppermost portion of the lateral membrane as well as interspersed with adherent junctions along a broad segment of the lateral membrane (Leach & Firth, 1992; Leach et al., 2000), and in freeze-fracture replicas they appear to be predominantly associated with the E and not the P-face (Muhleisen, Wolburg & Betz, 1989; Simionescu, Simionescu & Palade, 1976). There is an ample variability in TJ complexity and degree of tightness among different epithelial tissues and portions of the vascular tree (Bazzoni & Dejana, 2004b).

Epithelia Protect the Organism from the Environment

The importance of epithelia as a line of defense to external aggressors is easily illustrated by situations like burns or scraps where the disruption of the epithelial layer greatly increases the likelihood of a microbial infection. However, in mammals, the largest mucosal surface that provides an interface with the external environment is the intestinal epithelium. It provides protection against foreign antigens, toxins and molecules entering the body by the oral/enteric route. In a healthy state a minimal fraction of antigens crosses this defensive barrier, but during prematurity, exposure to radiation, chemotherapy or toxins the integrity of the TJ becomes compromised and immune responses to environmental antigens develop. In fact, a spectrum of diseases (e.g., ankylosing spondylitis, insulin-dependent diabetes mellitus, IgA nephropathy, multiple sclerosis, Celiac disease and inflammatory bowel disease) have in recent times been found to be associated with the intestinal presentation of environmental antigens due to abnormally high gastrointestinal TJ permeability (Fasano, 2001).

Therapeutic Agents Need to Cross Epithelia and Endothelia to Reach Their Site of Action

Although the uncontrolled flow of substances by the paracellular route may induce a pathological condition, the regulated and reversible passage of therapeutic agents through intercellular TJs may constitute a powerful tool for the delivery of drugs not absorbed through epithelial sheets that need to enter the systemic circulation in order to reach their site of action.

Drugs can cross the cells by two ways: the paracellular and the transcellular routes (Fig. 1*B*). The pathway chosen depends primarily on the physicochemical properties of each pharmaceutical agent. Hence lipophilic drugs traverse the biological membrane by the transcellular pathway, while hydrophilic ones cross the membrane through the paracellular route. The transcellular transport involves the sequential passage of molecules across the apical and basolateral epithelial membranes. When the molecule is not lipophilic, these steps can be mediated by channels, carriers and pumps. These proteins are highly specific for their transported solute and are also tightly regulated. Therefore, methods employing such transport systems have shown poor functioning with applied drugs. In contrast, the paracellular pathway, regulated by the TJ, has recently begun to be explored as a promising route for drug delivery.

The importance of the paracellular route in epithelial transport was well known since several decades ago. The renal tubules for example, reabsorb half of all sodium (Moe, Berry & Rector, 2000), 90% of all calcium (Friedman & Gesek, 1993) and 60% of filtered Mg^{2+} (Quamme & de Rouffignac, 2000) by passive diffusion through the paracellular pathway. Moreover, in vertebrates the intestine's capacity to absorb glucose by transcellular mediated pathways is inadequate to meet daily glucose intake, and requires absorption by solvent drag across intestinal TJs, secondary to active sugar and amino acid transport (Pappenheimer & Reiss, 1987; Pappenheimer, 1990). In fact, in several avian species, most glucose absorption appears to occur through the gut paracellular route (Chang & Karasov, 2004). This high intestinal permeability has the cost of allowing toxins to be absorbed from plant and animal material in the intestinal lumen. This vulnerability to hydrophilic toxins constrains avian food exploratory behavior, limiting the dietary niche and inducing compensatory conducts like ingesting specific substances that inhibit hydrophilic toxin absorption (Diamond, Bishop & Gilardi, 1999).

Although the importance of the paracellular route for transepithelial transport was recognized as early as the 1960s and 1970s, the design of strategies for regulating this pathway to enhance drug delivery is a new development. This situation is due to the fact that only recently the molecular composition and mechanisms of the TJ have begun to become unraveled.

Molecular Components of the TJs

The TJ is a multiprotein complex constituted by integral, adaptor and signaling molecules (Fig. 1*C*).

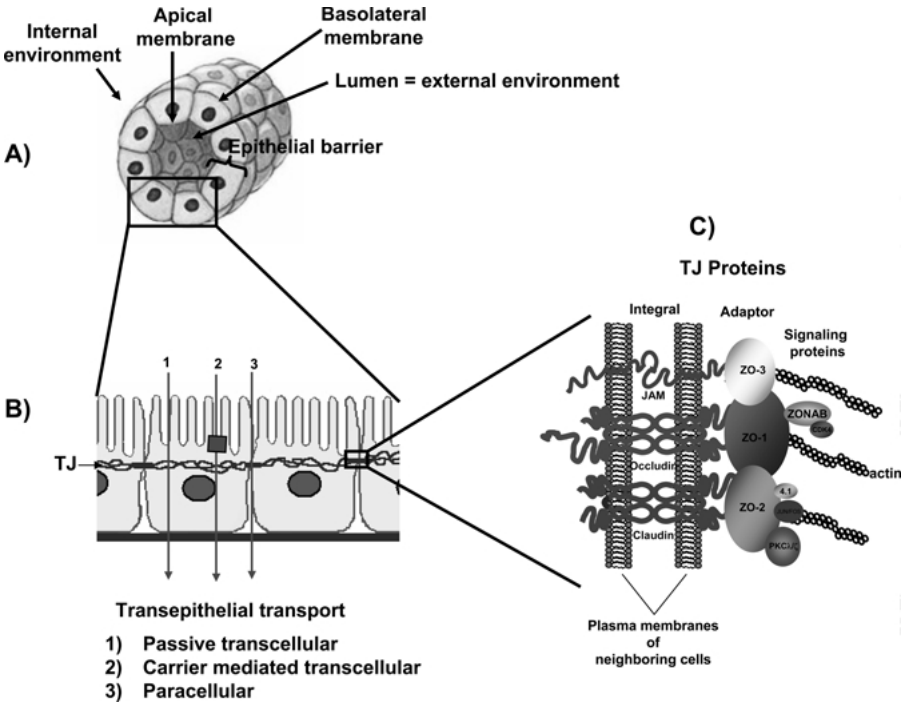

Fig. 1. Schematic representation of the epithelial barrier, the transepithelial pathways and the basic molecular organization of the TJ. (*A*) Epithelia form a polarized barrier that separates the luminal content of body cavities from the internal environment of the organism. No matter how deeply embedded in the body is an epithelial compartment, its content is considered as external environment for the organism. (*B*) Three pathways are available for transepithelial transport: (1) the passive transcellular pathway employed by hydrophobic compounds, (2) the transcellular carrier-mediated route, which is an active transport mainly limited to small molecules like sugars and amino acids, and (3) the paracellular route regulated by TJs. Hydrophilic molecules can cross epithelial and endothelial barriers by the paracellular pathway when the TJ is opened. (*C*) The TJ is constituted from a complex array of integral, adaptor and signaling proteins, some of which are illustrated in the diagram. Modulating the expression and protein-protein associations of TJ molecules allows the reversible opening of the paracellular pathway, facilitating drug delivery across epithelial and endothelial sheets.

Integral proteins mediate through their extracellular domains cell-cell adhesion of neighboring cells. The cytosolic regions of such proteins associate, in turn, with a set of adaptor molecules that work as scaffolds with binding regions for the actin cytoskeleton and for signaling molecules like kinases, phosphatases, small GTP binding proteins and transcription factors.

In this review we will not attempt to perform an extensive analysis of the molecular composition of the TJ, since several recent publications cover such a task (Gonzalez-Mariscal et al., 2003; Schneeberger & Lynch, 2004). Instead we will mention the main adaptor proteins and provide a brief description of the integral proteins of the TJ, since the latter constitute the target that most TJ modulators address to enhance drug delivery.

The TJ plaque is constituted by a wide array of adaptor proteins having one or more PDZ domains. These are sequences of 80–90 amino acids that form a hydrophobic groove that binds carboxyl terminal residues with the motif T/SXV or with a hydrophobic amino acid at the −2 position. PDZ domains also heterodimerize with other PDZ domains (Fanning & Anderson, 1999). Among the PDZ-expressing molecules of the TJ are the zonula occludens proteins ZO-1, ZO-2 and ZO-3 that belong to the family of membrane-associated guanylate kinase (MAGUK) homologues; the MAGUK inverted proteins MAGI −1, −2 and −3; the multi-PDZ domain proteins MUPP1 and PATJ and the partitioning-defective proteins PAR-3 and PAR-6.

At the TJ, three types of integral proteins have been identified: Occludin and the families of claudins and JAMs. The latter family is constituted by proteins that span the plasma membrane once, expose at the extracellular space two immunoglobulin (Ig) loops and have a cytoplasmic domain that ends with a PDZ binding motif. JAMs establish Ca^{2+}-independent cell-cell adhesion by interacting with several ligands in both homophilic and heterophilic manner (Hata & Hirabayashi, 2005; Miyoshi & Takai, 2005). JAMs are implicated in junctional assembly and stabilization, the regulation of paracellular permeability and leukocyte transmigration through endothelia and epithelia. JAMs are expressed in endothelial, epithelial and hematopoietic cells including monocytes, lymphocytes and red blood cells.

Although JAMs do not constitute TJ strands upon transfection (Itoh et al., 2001), they are crucial for TJ formation in epithelial cells. During the initiation of cell-cell contacts, nascent adherens junction (AJ) formed at the tips of filipodia and lamellipodia are enriched in nectins. These members of the Ig superfamily, characterized by containing three Ig domains (Takai & Nakanishi, 2003), form a microcluster that recruits E-cadherin, the integral protein of adherens junctions, to the lateral membrane, which in turn starts to expand. At the uppermost portion of this membrane, JAMs are then assembled and claudins and occludin follow shortly (Ebnet et al., 2000, 2001; Hirabayashi et al., 2003).

Both occludin and claudins are tetraspan proteins that traverse the plasma membrane four times and expose two loops towards the intercellular space. Upon transfection into L fibroblasts, occludin and claudins are capable of generating, albeit at a different degree of complexity, the appearance of filaments at the plasma membrane, which resemble those present at the TJ of epithelial cells (Furuse et al., 1998). The observation that antibodies against claudins and occludin (Fujimoto, 1995) specifically stain TJ filaments, further gave rise to the concept of TJ strands as heteropolymers integrated by occludin and a variety of claudins.

Occludin was initially proposed to play a functional role as a paracellular barrier molecule, since the expression of mutant and chimeric occludin perturbed the permeability of cultured epithelial cells (Balda et al., 1996, 2000) and *Xenopus* embryos (Chen et al., 1997). This interpretation has. however, been challenged by the unexpected results obtained in occludin knockout mice, where TJs do not appear to be affected either morphologically or functionally (Saitou et al., 2000; Schulzke et al., 2005). Instead, histological abnormalities such as chronic inflammation and hyperplasia of the gastric epithelium, bone thinning and a progressive accumulation of calcium and phosphorous deposits in the cerebellum and basal ganglia, have been detected in occludin null animals. These results hence suggest that occludin is not essential for the formation of TJ strands, probably due to the presence of other TJ integral proteins capable of developing the TJ barrier function, but yet indicate that the expression of occludin is important for the regulation of cellular differentiation in certain organs.

Claudins are members of a multigene family found across a broad range of multicellular organisms. More than twenty members have been identified, each displaying a tissue-specific distribution. A growing amount of evidence depicts claudins as channels responsible for the selective size and charge properties of the paracellular pathway. For example:

1) The extracellular loops of claudins exhibit charged residues (Mitic & Van Itallie, 2001), which upon modification alter the ionic selectivity of the TJ. Thus, when a negatively charged residue of the first extracellular loop of claudin 4 is switched for a positive one, the transfected cells show an increased paracellular Na^+ permeability, while substituting positive for negative charges in the first extracellular domain of claudin 15 changes the paracellular charge selectivity from Na^+ to Cl^- (Colegio et al., 2002).
2) The first extracellular loop of claudins defines the ionic selectivity of the TJ paracellular pore. Hence, chimeras expressing the first or both extracellular domains of claudin 4 on claudin 2, behave electrophysiologically more like claudin 4 than like claudin 2, as they increase TER several-fold and profoundly decrease the permeability of Na^+ relative to Cl^- (Colegio et al., 2003).
3) The transfection of claudins modifies the paracellular properties of the epithelia, although the cell background should be considered, since the role of each claudin in the paracellular transport is more easily detectable when observed in monolayers exhibiting the opposite charge selectivity. Therefore, the introduction of claudins 4 and 11 in the cation-selective MDCK cells decreases the paracellular Na^+ permeability, whereas claudin 2 and 15, which behave as cationic pores, exert no effect in this cell line. In contrast, in the anion-selective LLC-PK1 cells, claudins 2 and 15 increase the paracellular Na^+ permeability, while claudin 4 and 11 show no effect (Van Itallie, Fanning & Anderson, 2003).
4) In humans, mutations in claudin 16 are present in familial Mg^{2+} and Ca^{2+} wasting diseases (Simon et al., 1999; Muller et al., 2003; Ikari et al., 2004), strongly suggesting that this claudin constitutes a Mg^{2+} and Ca^{2+} channel. In normal individuals claudin 16 is found at the ascending thick limb of Henle (Weber et al., 2001), where up to 60% of the renal filtered Mg^{2+} is reabsorbed by passive diffusion through the paracellular pathway (Quamme, 1997).
5) Mutant mice deficient in claudin 1 die soon after birth due to water loss through the skin, hence suggesting a crucial participation of claudin 1 in the establishment of the skin barrier (Furuse et al., 2002). A similar death is encountered by mice overexpressing claudin 6 (Turksen & Troy, 2002), suggesting that the presence of this claudin instead facilitates the paracellular flow of water through the skin. Claudin 5-deficient mice display a blood brain barrier that allows the passage of a 562 Da paracellular marker but still restricts the flow of an

1863 Da marker, indicating that claudin 5 plays a role in size discrimination at the TJ (Nitta et al., 2003). The absence of claudin 11 gives rise to a deafness phenotype in adult knockout mice (Gow et al., 2004; Kitajiri et al., 2004). In such animals the endolymph of the cochlea maintains its characteristic high K^+ concentration but displays a lower endocochlear potential. This situation inhibits cochlear hair cells to transduce acoustic stimuli to electrical signals and hence produces deafness. The origin of this condition is due to the altered phenotype of the basal cells of the stria vascularis, located adjacent to the endolymph compartment, that no longer exhibit TJs and allow the free flow of paracellular tracers. In humans, mutations of the gene encoding claudin 14 cause profound deafness (Wilcox et al., 2001) and claudin 14 null mice have a normal endocochlear potential but are deaf due to degeneration of the cochlear outer hair cells. The localization of claudin 14 at the hair cells of normal animals, together with the observation that in MDCK cells, claudin 14 decreases cation permeability, suggests that the deafness encountered in claudin 14 null mice is due to the absence of a cation-restrictive barrier capable of maintaining a proper ionic composition of the fluid surrounding the basolateral surface of the outer hair cells (Ben Yosef et al., 2003).

Taken together, these results highlight the importance of claudins in regulating the selective flow of ions and molecules of different charge and sizes through the TJ.

Strategies to Open the TJ that Could Enhance Paracellular Drug Delivery

A variety of strategies designed to open the TJ are starting to be explored in order to enhance the paracellular passage of drugs through epithelia and endothelia (Table 1). To facilitate the analysis of the assays employed we will mention that the degree of junctional sealing is usually determined by measurements of transepithelial electrical resistance (TER) and by the paracellular flow of non-ionic tracers such as mannitol and dextrans of different sizes. These determinations are performed in mature monolayers with sealed TJs (Cereijido et al., 1978a; Martinez-Palomo et al., 1980b) and in cultures that, after being incubated for several hours in a Ca^{2+} depleted medium, are transferred to Ca^{2+}-containing medium. This protocol, designed to analyze the process of TJ assembly, is frequently referred to as Ca-switch (Gonzalez-Mariscal, Chavez & Cereijido, 1985).

Peptides Designed to Uncouple the Interaction between the Integral Proteins of the TJ at the Intercellular Space

When the molecular identity of the integral proteins of the TJ was unraveled in the late nineties, several stratagems were designed to study their role as sealing elements of the TJ. Among them, one designed by Gumbiner and Wong, later proved to be particularly important for the field of drug delivery (Wong & Gumbiner, 1997). It was hypothesized that if cell-cell contacts were being established at the paracellular space by the homotypic association of the extracellular loops of occludin, it would be possible to compete such interaction with the addition of synthetic peptides corresponding to the extracellular loops of occludin. In effect, the peptide homologous to the second loop of chicken occludin, but not to the first, reversibly reduced in a *Xenopus laevis* kidney cell line (A6) the TER and increased the paracellular flux of non-ionic tracers in a dose-dependent manner. This experiment became a turning point, since it proved for the first time that segments homologous to the extracellular domains of TJ proteins could be used to manipulate epithelial permeability.

Later, shorter peptides corresponding to residues 100 to 108 or 109 of the first loop of chicken occludin, also proved to be useful to impair TJ resealing triggered by Ca^{2+} in A6 cells (Lacaz-Vieira et al., 1999), and peptides containing amino acids 90–112 or 113–135 of the first loop of human occludin inhibited cell adhesion induced by occludin expression in rat fibroblasts (Van Itallie & Anderson, 1997). The reason why the longer peptide, analogous to the complete first loop of occludin, was ineffective in A6 cells, whereas shorter peptides of this loop effectively altered cell adhesion in A6 cells and other cell lines, is unclear. However, it is possible that the differences arose by the employment of a long peptide emulating chicken occludin, given that the amino-acid sequence of the first occludin loop in mammals diverges around 50% from that of chicken, while the mammalian ones are closely related to each other (approximately 90% identity) (Ando-Akatsuka et al., 1996).

More recently, a similar system was proposed to examine the molecular interactions of TJ integral proteins. Thus, peptides emulating the second loop of human occludin have selectively associated with occludin, claudin 1 and JAM-A and impeded the recovery of functional TJs induced by Ca^{2+} (Nusrat et al., 2005). This study thus demonstrated that with a peptide corresponding to a segment of one TJ protein, in this case occludin, it is possible to alter homologous and heterologous protein-protein interactions present at the intercellular space of the TJ.

Other studies have demonstrated that a 4-residue peptide found in the second extracellular domain of

Table 1. Strategies to open the TJ that could enhance paracellular drug delivery

Strategy		Effect	Reference
Peptides corresponding to external portions of TJ integral proteins	Occludin	Diminished TER and increased paracellular flux of nonionic traces in monolayers with sealed TJ	Wong & Gumbiner, 1997
		Inhibition of TER development in a Ca-switch assay	Lacaz et al., 1999
		Inhibition of cell aggregation induced by occludin expression in fibroblasts	Van Itallie & Andersen, 1997
		Inhibition of TER development and increased paracellular flux of nonionic traces in a Ca-switch assay	Nusrat et al., 2005
		Inhibition of cell aggregation induced by occludin expression in fibroblasts	Blaschuck et al., 2002
	JAM	Diminished rate of TER in a Ca^{2+} restitution assay	Liang et al., 2000
Specific degradation of the mRNA for TJ proteins	ZO-1 antisense oligonucleotides	Diminished fluid flow resistance induced by dexamethasone in cells from Schlemm's canal and the trabecular meshwork of the human eye	Underwood et al., 1999
	siRNAs for occludin	Diminished peak TER values attained in a Ca-switch assay but no effect on final steady state TER values	Yu et al., 2005
	siRNAs for Claudin 4	Decreased TER and increased dextran permeability in mature epithelial monolayers	Dutzar et al., 2005
Bacterial toxins	*Clostridium difficile* enterotoxins	Displaced TJ proteins, diminished TER and increased paracellular flux of solutes	Hecht et al., 1988 and 1992
	Esoherichia coli enterotoxins	TJ disruption and increased paracellular permeability	Atisook et al., 1990
		Occludin dephosphorylation and shift from the TJ to the cytosol	Simonovic et al., 2000
	Vibrio cholerae ZOT enterotoxin	TJ opening and increased intestinal absorption of insulin, IgG and other therapeutic agents	Fasano et al. 1995, 1997; Marinaro et al., 2003; Salama et. al., 2005
Proteins from enteric pathogens that cleave TJ proteins	*Clostridium perfringes* enterotoxin (CPE)	Removal of specific claudins from TJ filaments	Fujita et al., 2000; Mc Clane & Singh 2001
	Ochratoxin A	Removal of specific claudins from TJ filaments and decreased TER	Maresca et al., 2001; Mc Laughin et al., 2004
	Vibrio cholerae Haemagglutinin protease	Occludin degradation	Wu et al., 2000
Viral proteins	Rotavirus VP8	Decreased TER in a reversible and dose dependent manner that allows intestinal absorption of insulin	Nava et al., 2004
	Rotavirus NSP4	Decreased TER and increased paracellular passage of dextran	Tafazoli et al., 2001
Endogenous epithelial proteins	Zonulin	TJ opening	Wang et al., 2000
Detergents/surfactants	Sodium caprate	Increase paracellular transport of mannitol and dextran	Lindmark et al., 1998; Tomita et al., 1995
	Saponin, dipotassium glycyrrhizinate, 18β-glycyrrhetinic acid	TJ widening	Cho et al., 2002
	Protamine	Increased or diminished TER depending on the tissue type	Peixoto & Collares-Buzato 2005
Polycations	Chitosan	Reversible and dose-dependent decrease in TER, increased mannitol permeability and junctional displacement of TJ proteins	Dodane et al., 1999

occludin in all mammalian species, is sufficient to inhibit the establishment of endothelial barriers in vitro and in vivo. This LYHY motif, named cell adhesion recognition (CAR) site of occludin (Blaschuk et al., 2002), however, requires, in order to be effective, to be given in a cyclic form generated by introducing a pair of Cys at the peptide extremes (Oshima et al., 2003).

With the purpose of unraveling the process of TJ assembly, JAM proteins have been added to confluent epithelial cells in a resistance -recovery assay after calcium depletion. The results obtained have been conflicting. Thus, when the JAM employed (huJAM-Fc) was produced in insect cells, a significant lowering of the rate of TER values was detected (Liang et al., 2000). In contrast, when the same JAM was coupled to alkaline phosphatase and expressed as a secreted protein in COS-7 cells, no inhibition of TER recovery was generated (Liu et al., 2000). This suggests that the latter construction does not adequately recapitulate the full structural requirement of JAM capable of interfering with the homo- or heterotypic interaction of the protein at the TJ.

As a result of all these observations peptides from 4 to 25 contiguous amino acids of the extracellular domains of JAMs, occludin and claudins are presently being claimed in patent offices around the world, to enhance mucosal delivery of therapeutic agents (Blaschuck & Gour B.J.,1999; Blaschuck, Symonds & Gour B.J., 2000; Anderson & Van Itallie, 2002; Turksen, 2003; Quay, 2004).

Modifications in TJ Peptides to Improve Drug Delivery

Since pharmacological agents are generally administered to the luminal side of epithelia, a prerequisite of their functionality is to be active from the apical surface. To achieve this goal a peptide analogous to residues 90–113 of the first loop of human occludin, with effect only when applied at the basolateral surface, was conjugated on its amino terminus to a lipoamino acid [H_2N-CH ($C_{12}H_{25}$)-COOH], previously shown to inhibit peptide degradation in intestinal cell lines (Yamamoto, 1998; Toth et al., 1999). The occludin peptide was now rendered active when applied to the apical surface (Tavelin et al., 2003). This conjugate has the advantages of inserting into the apical cell membrane and releasing the active peptide through the action of the brush border peptidases at sites adjacent to the TJ. This situation apparently limits the degradation of the released peptides by apical peptidases and reduces peptide aggregation. This effect is crucial for the rapid opening of TJs, since if the kinetics is too slow the peptide may be degraded or washed from the intestinal surface before it is effective, or the co-administrated drug will have been transported away from the site of action of the peptide. The D isomer of the conjugate further appears to be the more desirable form, since it displaye a rapid and transient effect, while the L isomer gave a more sustained increase in permeability. This is particularly important, as a short duration of the action will limit the unrestricted access of potentially toxic agents present in the intestinal lumen and would also reduce the potential risk of autoimmune responses. Therefore, the lipoamino-acid conjugates of TJ peptides represent a promising class of TJ modulators specially suited for drug delivery.

The combination of TJ peptides with other mucosal delivery-enhancing agents might further increase their effectiveness. Examples of agents that could be employed are: an aggregation inhibitor agent, a pH control agent, a degradative enzyme inhibitor, a mucolytic or mucus-clearing drug, a ciliostatic, a membrane penetration-enhancing agent or a stabilizing delivery carrier.

Silencing the Expression of Specific TJ Proteins

An alternative approach for regulating the paracellular transport of molecules across epithelial and endothelial cells is the specific degradation of mRNA of individual TJ proteins. An example of these studies was done in human eye endothelial cells derived from Schlemm's canal (SCE) and the trabecular meshwork (TM). These cultures were used to study the pathophysiology of steroid glaucoma, since glucocorticoid treatment increases the resistance encountered by aqueous humor when it exits the eye. As dexamethasone induced an increased expression of the TJ adaptor protein ZO-1, antisense phosphorothioate oligonucleotides for ZO-1 were employed. These diminished the amount of ZO-1 and the increased fluid flow resistance induced by the steroid, hence demonstrating the feasibility of regulating the paracellular flux by silencing ZO-1 expression (Underwood et al., 1999).

A more recent procedure developed for the specific silencing of TJ proteins is the employment of small interfering RNAs (siRNAs). Following such a strategy, occludin expression has been successfully knocked down in cultured epithelial cells (Yu et al., 2005). In these monolayers, occludin silencing has a low impact on the paracellular route as it 1) diminishes the peak TER levels attained in a Ca-switch protocol and in TJ de novo formation studies, but exerts no effect either on the final steady-state TER values achieved or in the TER displayed in mature monolayers; 2) does not increase the paracellular flux of 3 and 10 kDa dextrans and only produces a small increase in mannitol flux if hydrostatic pressure is applied. These results are reminiscent of those obtained in occludin knockout mice, and suggest that when occludin is not available, the cell is capable of

forming functional TJs, probably by incorporation of other proteins with functions similar to those of occludin. Therefore, silencing occludin expression appears not to be a promising tool for paracellular drug delivery. These results are in sharp contrast with the aforementioned ones obtained with the occludin peptides. We suspect that the difference lies in the fact that the occludin peptides are applied to wild-type monolayers where occludin is a conspicuous constituent of TJs.

Preliminary studies with siRNA for several claudins have been performed in epithelial respiratory cells. In them, claudins 3 and 4 are only expressed in the differentiated state. The siRNA knockdown of claudin 4 inhibited the formation of TJs and resulted in a significant TER decrease and an increase in dextran permeability (Dutzar et al., 2005), that could in principle be employed to enhance paracellular drug delivery. However, the major challenges for this approach reside in 1) ensuring an efficient intracellular siRNA delivery, since siRNA cannot passively diffuse through the cell membrane or traverse the paracellular pathway, and 2) the period of 2–3 days required to obtain protein silencing after siRNA is delivered intracellularly.

Toxins and Proteins Derived from Microorganisms Capable of Opening the TJ

Epithelia constitute the first line of defense of the organism against microorganisms. Throughout evolution pathogens have devised several ways to disrupt epithelial TJs in order to gain access to nutrients from their hosts. To date, this field of research has focused primarily on the effect of enteric pathogens. Some of these microorganisms open the paracellular pathway by affecting specific TJ proteins or by altering the cell cytoskeleton, while the mechanism employed by others remains unknown. The capability of these compounds to open TJs has recently begun to be explored as a strategy for facilitating drug delivery across epithelial and endothelial sheets.

Bacterial Toxins that Affect the TJ through a Rearrangement of the Actin Cytoskeleton

Clostridium difficile Enterotoxins. Among the best-known bacteria that alter TJs by disrupting the actin cytoskeleton are *Clostridium difficile*, and *Escherichia coli*. The former elaborates toxins A and B that monoglucosylate the Rho family of proteins Rho, Rac and Cdc42, rendering them inactive (Just et al., 1994, 1995). As a consequence, filamentous actin is degraded, cells round up, the TJ proteins are displaced, TER diminishes and the paracellular flux of solutes increases (Hecht et al., 1988, 1992).

Toxin A has further been found to activate protein kinase C α and β to redistribute ZO-1 (Chen, Pothoulakis & LaMont, 2002).

E. coli Enterotoxins. *E. coli* induces in host cells the accumulation of cytoskeletal proteins underneath its site of attachment, and inserts through its secretion apparatus effector molecules into the invaded cell. In enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC) these inserted proteins stimulate the phosphorylation of myosin light chain (MLC) by its kinase (MLCK), generating cytoskeletal contraction (Yuhan et al., 1997). This tension in turn induces TJ disruption and increases paracellular permeability (Atisook, Carlson & Madara, 1990). It has been further observed that EPEC infection generates occludin dephosphorylation and its shift from the TJ to the cytosol (Simonovic et al., 2000). Different isoforms of PKC also participate in the signaling cascade triggered by EPEC and EHEC infection (Philpott et al., 1998; Savkovic, Koutsouris & Hecht, 2003).

Zot, the Vibrio *Cholera Toxin and the Host Zonulin System.* From a therapeutic point of view, the employment of the above-mentioned bacterial toxins to enhance drug delivery seems complicated as their effects are mediated by general and complex intracellular pathways that, once activated, might be difficult to control in order to obtain reversible opening of the TJ. In spite of this, Zot (Zonula occludens toxin) a *Vibrio cholera* toxin, has efficiently been employed to enhance drug delivery. This toxin was identified while searching for factors responsible for the residual diarrhea generated with *V. cholera* vaccines of cholera toxin (Baudry et al., 1992). Zot is a 44.8 kDa polypeptide, present at the outer membrane of *V. cholera*. After cleavage, a carboxyl 12 kDa fragment, responsible for the biological effect of the toxin, is excreted (Uzzau, Cappuccinelli & Fasano, 1999). Zot action is tissue-specific. Thus it is active on the mucosal side of epithelial cells of the small intestine and on endothelial cells but not on colonocytes or renal epithelial cells. In enterocytes the number of Zot receptors decreases along the villous crypt axis, suggesting that the expression of the receptor is associated with epithelial differentiation (Fasano et al., 1997).

Zot induces a PKC_{α}-dependent polymerization of actin microfilaments that leads to opening of the TJ (Fasano et al., 1995). The reason why Zot does not induce an uncontrolled opening of the paracellular pathway and instead displays a reversible one that can be employed for drug delivery, may lie in the fact that it has a eukaryotic analogue and hence epithelial and endothelial tissues might count on a mechanism to control the extent of its action. Employing anti-Zot antibodies and Ussing chamber assays, an endogenous molecule named Zonulin was found to reversibly open the TJ after engagement to the same receptor activated by Zot (Wang et al., 2000). Zonulin has an amino-terminal binding-motif

of 8 amino acids that is structurally and functionally similar to the Zot-binding motif. Zonulin has been detected in the human intestine, heart and brain. The physiological role of zonulin is starting to be unraveled, but it has tentatively been proposed to serve during tissue morphogenesis to facilitate the passage of molecules between body compartments, while in adults it could participate in the transit of leukocytes between the bloodstream and the intestinal lumen. Another interesting possibility has been raised by the observation that the presence of enteric microorganisms in the small intestine, irrespective of their pathogenic traits or viability, induce the luminal secretion of zonulin, hence TJs are opened and water is secreted into the intestinal lumen, according to the hydrostatic pressure gradients, and bacteria are flushed out of the small intestine (Fasano, 2001). Since to date the main source for zonulin purification is human cadaver, Zot has alternatively been employed in drug delivery assays. The efficacy of Zot has been demonstrated by the increased intestinal absorption of insulin, immunoglobulin G, and several other therapeutic agents (Fasano & Uzzau, 1997; Marinaro, Fasano & De Magistris, 2003; Salama et al., 2005). The passage of molecular weight markers and chemotherapeutic agents across brain microvessels has also been reported (Karyekar et al., 2003).

Proteins Derived from Enteric Pathogens that Cleave TJ Proteins

Fragilysin

Other enteric pathogens induce disruption of TJs as a result of protein cleavage by proteases. For example, the diarrhoeic strain of *Bacteroides fragilis* produces a zinc-binding metalloprotease known as fragilysin that decreases TER and increases paracellular permeability (Chambers et al., 1997; Obiso Jr., Azghani & Wilkins, 1997). The target of this protease is the extracellular domain of E-cadherin (Wu et al., 1998), the protein responsible for establishing cell-cell contact at the adherens junction (AJ). Therefore, fragilysin opens the paracellular pathway through the disruption of the AJ.

CPE and Ochratoxin A

Certain strains of the anaerobic bacterium *Clostridium perfringens* produce an enterotoxin (CPE) responsible for food-borne, gastrointestinal illness and antibiotic-associated diarrhea. When present in the small intestinal lumen, these bacteria multiply and sporulate. During the latter process CPE is released into the intestinal lumen, where it binds to a receptor. Interestingly it appears that the TJ proteins, claudins 3, 4, 6, 7, 8 and 14 function as CPE receptors (Fujita et al., 2000; McClane & Singh, 2001). Following CPE membrane binding, a. series of SDS-resistant complexes of 90, 135, 155 and 200 kDa are formed. The 155 kDa complex apparently forms pores in the apical membrane that leads to massive membrane permeability alterations and lethal cell damage that initiates the CPE-induced fluid and electrolyte loss (Hardy et al., 1999). This complex next interacts with occludin to integrate a larger 200 kDa complex (Singh et al., 2000) that rapidly disintegrates TJs, contributing secondarily to the diarrheic symptoms of CPE-induced illness. The C-terminal portion of CPE (C-CPE), which lacks cytotoxic activity and has instead the claudin-binding domain, induces disruption of TJ fibrils and removal of specific claudins from TJs when applied to the basolateral surface of polarized epithelial cells (Sonoda et al., 1999). A somewhat similar effect is observed with the ochratoxin A, a mycotoxin that contaminates cereals and animal feed and causes nephropathy, intestinal inflammation, diarrhea, and increased bacterial translocation. This toxin induces a decrease in TER concomitant with the specific removal of claudins 3 and 4, but not claudin 1 from the cell membranes (Maresca et al., 2001; McLaughlin et al., 2004).

Hemagglutinin Protease (HA/P) of Vibrio Cholera

Another bacterial toxin that targets TJ proteins is the hemagglutinin protease (HA/P) of *Vibrio Cholera*. When this zinc metalloprotease is applied to the apical surface of epithelial monolayers, TER diminishes and occludin is degraded at two extracellular sites adjacent to the membrane. The hemagglutinin/protease (HA/P) effect is dose- and time-dependent (Wu, Nybom & Magnusson, 2000). ZO-1 and the actin cytoskeleton also become disrupted, although ZO-1 was not degraded by HA/P, suggesting that occludin degradation perturbs the ZO protein complex, ultimately leading to a reorganization of the actin cytoskeleton.

Together these results suggest that TJ protein degradation induced by microbial toxins is a common mechanism of pathogenesis. The employment of these toxins to facilitate the passage of therapeuticals through the paracellular route is hampered by the fact that TJ proteins are being degraded, whereas a swift and reversible opening of the TJ would be preferred in order to avoid the passage of potentially toxic compounds present in the intestinal lumen and to avoid the risk of developing autoimmune diseases. Instead, bacterial toxins that degrade TJ proteins are better suited for cancer treatment, considering, for example, that in some cancers (e.g., breast cancer) claudins 3 and 4 are overexpressed. Treatment with CPE has already been shown to lyse

metastatic cancer of the breast, brain and bone (Kominsky et al., 2004; Sukumar & Kominsky, 2003).

Viral Proteins that Open the TJ by Mechanisms not yet Defined

Rotavirus VP8

Rotaviruses infect mature enterocytes of the villi of the small intestine as well as a wide variety of renal and intestinal cell lines. Rotaviruses utilize integrins as cell receptors. However, in polarized epithelia, integrins are located at the basolateral membrane beneath the TJ, while rotaviruses that have entered the body through oral ingestion are found in the lumen of the intestine. This observation led to a search of proteins present in the rotavirus surface that could open the TJ in order to allow viral particles to reach their integrin receptors. VP8, a protein generated by the proteolytic cleavage of a spike-forming protein named VP4, which protrudes from the outermost layer of the rotavirus capsid, has been found to decrease TER of epithelial monolayers in a reversible and dose-dependent manner (Nava et al., 2004). Moreover, VP8 perturbs the fence function of the TJ, allowing the displacement of basolateral proteins to the apical surface and vice versa, hence permitting the movement of integrins to the apical surface where the rotaviruses are concentrated.

In rats, the oral administration of VP8 with insulin allows the absorption of the therapeutic agent in the intestine, without provoking diarrhea or fever. Therefore VP8 has been proposed as an enhancer for drug delivery. The mechanism of action of VP8 is not yet resolved, but the presence in VP8 of several regions similar to segments present in the external loops of occludin and claudins strongly suggests that it may act through a mechanism similar to that triggered with peptides against integral TJ proteins.

Rotavirus NSP4

This nonstructural protein of rotavirus is an enterotoxin capable of inducing diarrhea in young mice and is responsible for an increase in cytosolic Ca^{2+} through phospholipase C activation and IP_3 release (Dong et al., 1997). The apical, but not the basolateral administration of NSP4 causes a reduction in TER, redistribution of filamentous actin and an increase in the paracellular passage of dextran. Although the effect of NSP4 is reversible, its potential as a drug delivery enhancer will have to be explored with higher doses, since the induced decrease in TER happens only after incubations of 20 to 30 h (Tafazoli et al., 2001).

Other Compounds Capable of Enhancing Drug Delivery

More than two decades ago, the role of Ca^{2+} chelators on TJ opening was described (Cereijido et al., 1978b, 1980; Martinez-Palomo et al., 1980a). However, their utility for promoting the paracellular absorption of molecules is limited, since Ca^{2+} depletion induces drastic cellular changes that include disruption of the actin filaments and adherent junctions and diminished cell adhesion (Cereijido et al., 1980).

Therefore, other numerous compounds with diverse chemical structures have been tested for their potential to increase the absorption of drugs and polypeptides. Many of these drug enhancers are detergents/surfactants that increase the transepithelial transport of drugs by altering the basic structure of the lipid bilayer, making the plasma membrane more permeable. These compounds, which include bile salt, fatty acids, medium chain glycerides and acyl carnitines among others, are capable of lysing biological membranes by a mechanism that includes lipid solubilization followed by protein denaturation and solubilization (Kirkpatrick & Sandberg, 1973; Kirkpatrick, Gordesky & Marinetti, 1974). The lytic potential of these agents may therefore induce exfoliation of the epithelium and might irreversibly compromise its barrier function. Some surfactants like fatty acid sodium caprate and long-chain acylcarnitines that improve drug absorption, do not generate an apparent damage to the epithelia (Hochman & Artursson, 1994). However, when the cytotoxicity of sodium caprate, sodium deoxycholate, and dipotassium glycyrrhizinate was tested, only the latter was found not to be cytotoxic (Sakai et al., 1998). Therefore, until more detergent/surfactants prove to enhance transepithelial drug delivery without generating epithelial damage, the alternative approach of selectively opening the paracellular pathway remains an option worth exploring.

Some detergents/surfactants also increase the permeability through the paracellular pathway. For example, sodium caprate increases paracellular transport of mannitol and dextrans via phospholipase C activation, upregulation of intracellular Ca^{2+} and actin-myosin filament contraction, which leads to TJ opening (Lindmark, Kimura & Artursson, 1998; Tomita, Hayashi & Awazu, 1995). Other enhancers like saponin, dipotasium glycyrrhizinate, 18 beta-glycyrrhetinic acid, sodium caprate and taurine improve paracellular transport by widening the TJs (Cho et al., 2002).

In the early eighties it was discovered that a positively charged protein, isolated from the urine of a Fanconi's syndrome patient, induced a dose-dependent increase in TER of the *Necturus* gallbladder (Alavi et al., 1983). Since then the effect of several polycations has been studied on epithelial

permeability. Polymorphonuclear leukocytes release a number of such proteins (e.g., major basic protein, eosinophil cationic protein and heparin binding protein), which are proposed to contribute to the pathogenesis of the inflammatory condition that increases vascular permeability and edema at sites of inflammation (Henson & Johnston Jr., 1987; Gautam et al., 2001; Jones, Paul & Page, 2001). Some polycations that have been used to reproduce the charge-dependent effect of endogenous cationic proteins on epithelia are protamine, poly-L-lysine and chitosan. Protamine elicits diverse and conflicting results, as it may increase or decrease the TER, depending on the tissue type or the degree of leakiness of the cell line. In high-resistance MDCK cells (strain I), protamine perturbs the epithelial barrier function by affecting the actin cytoskeleton and reducing the expression of the TJ proteins occludin and claudin-1 (Peixoto & Collares-Buzato, 2005). Chitosan, a mucoadhesive polymer obtained by the N-deacetylation of chitin, a polysaccharide found in the exoskeleton of shellfish like shrimps or crabs, causes a reversible and dose-dependent decrease in TER, an increased mannitol permeability and junctional displacement of TJ proteins (Dodane, Amin & Merwin, 1999). Chitosan and its derivatives effectively increase the permeation of macromolecules across mucosal epithelia by TJ opening (Thanou et al., 2000).

Concluding remarks

The discovery of the molecular constituents of the TJ has raised the possibility of manipulating its opening and closure. This has in turn given the opportunity of employing the paracellular pathway as an effective route for drug delivery. Transit through this route has the latent risk of introducing into the organism toxins contained in the epithelial lumenal compartments and of triggering autoimmune diseases. However, the possibility of significantly enhancing the delivery of therapeutic agents by opening the paracellular route of epithelial and endothelial sheets is of such importance that it is expected to trigger, in the years to come, a variety of strategies that will seek to reduce the risks derived from the transient opening of TJs.

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