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Review

Crosstalk of tight junction components with signaling pathways

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Abstract

Tight junctions (TJs) regulate the passage of ions and molecules through the paracellular pathway in epithelial and endothelial cells. TJs are highly dynamic structures whose degree of sealing varies according to external stimuli, physiological and pathological conditions. In this review we analyze how the crosstalk of protein kinase C, protein kinase A, myosin light chain kinase, mitogen-activated protein kinases, phosphoinositide 3-kinase and Rho signaling pathways is involved in TJ regulation triggered by diverse stimuli. We also report how the phosphorylation of the main TJ components, claudins, occludin and ZO proteins, impacts epithelial and endothelial cell function.
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Keywords: Tight junction; Claudin; Occludin; ZO-1; MAPK; PI3K; AKT; Rho; MLCK; PKC; PKA; PKG

Abbreviations: ANP, atrial natriuretic peptide; ARPE-19, human retinal pigment epithelial cell line; AsPC-1, human pancreatic cancer cell line; BAEC, bovine aortic endothelial cells (primary culture); BBB, blood–brain barrier; BPAEC, bovine pulmonary artery endothelial cell line; BRB, blood retinal barrier; C3, transferase from *Clostridium botulinum*; Caco-2, human colonic adenocarcinoma cell line; CAPAN-2, human pancreas epithelial cell line; CK2, casein kinase 2; Con8, rat mammary tumor epithelial cell line; CRIB, Cdc42 and Rac interaction binding; CRM1, chromosomal region maintenance 1; 94D, mouse cortical collecting Duct epithelial cell line; DAG, diacylglycerol; DHT, dihydrotestosterone; DiC8, phosphatidyl inositol 3,4,5-triphosphate; E_2, estradiol; EcN, *Escherichia coli* Nissle; ECT_2, A guanine nucleotide exchange factor; EGF, epidermal growth factor; EGTA, ethylene glycol tetraacetic acid; EHEC, enterohemorrhagic *Escherichia coli*; EMT, epithelial mesenchymal transition; EPEC, enteropathogenic *Escherichia coli*; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; FF, freeze–fracture; FGF, fibroblast growth factor; FHHNC, familial hypomagnesemia with hypercalciuria and nephrocalcinosis; GC, guanylate cyclase; GEF-H1, A guanine nucleotide exchange factor; GPI, glycosyl phosphatidyl inositol; GSK-3β, glycogen synthase kinase 3 beta; HBMEC, human brain microvascular endothelial cell line; HepG2, hepatic cell line; HGF, hepatocyte growth factor; HMEC, human dermal microvascular endothelial cell line; HPAEC, human pulmonary artery endothelial cell line; HT-29, human colon adenocarcinoma cell line; HUVEC, human umbilical cord endothelial cell line; ICM, inner cell mass; IEC-6, rat small intestine epithelial cell line; IFNγ, interferon gamma; IL-17, interleukine 17; JNK, c-Jun N-terminal kinase; LC, low calcium (1–5 μM Ca^{2+}); Lgl, lethal giant larva; LLC-PK1, pig renal cell line; LPA, lysophosphatidic acid; MAGUK, membrane-associated guanylate kinase; MAPK, mitogen-activated protein kinase; MARCKs, myristoylated alanine-rich C kinase susbstrate; MDCK, Madin–Darby canine kidney cell line; 2ME, 2 methoxyestradiol; ML7, 1-(5-iodonaphthalene 1-sulfonyl)-1H-hexadydro-1,4-iazepine HCl; ML9, 1-(5-cloronaphthalene 1-sulfonyl)-1H-hexadydro-1,4-diazepine HCl; MLC2, regulatory myosin light chain; MYPT, myosin phosphatase target subunit; NC, normal calcium (1.8 mM Ca^{2+}); NES, nuclear export signal; NLS, nuclear localization signal; NO, nitric oxide; NOS, nitric oxide synthase; N-WASP, neuronal Wiskott Aldrich syndrome protein; OmpA, outer membrane protein A; PAR-2, protease-activated receptor 2; PB1, Phox and Bem1p; PC1, Hamster pancreatic ductal carcinoma cell line; PHAII, pseudohypoaldosteronism type two; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PKG, protein kinase G; PLC γ, phospholipase C gamma; PLGF-1, placental growth factor 1; PLF, polyp-like foci; PMA, phorbol 12-myristate 13-acetate; POI, post-flight orthostatic intolerance; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; REC, retinal endothelial cells; RLE, rat lung endothelial cell line; ROMK, apical potassium channel; ROS, reactive oxygen species; SARA, Smad anchor for receptor activation protein; SGLT1, sodium glucose cotransporter; T-84, human colorectal carcinoma cell line; TAL, thick ascending limb of Henle; TEM, transmission electron microscopy; TER, transepithelial electrical resistance; TGFβ, transforming growth factor beta; TJ, tight junction; TLR2, Toll-like receptor 2; TNFα, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; VAC, vacuolar apical compartment; VE-cadherin, endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor; WB-F344, rat liver epithelial cell line; WNK, with no lysine kinase; Zot, zonula occludens toxin

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1. Introduction

Epithelia are the frontier between the internal milieu of the organism and the external environment constituted either by the air or water that surrounds the individual in case of the skin, or the content of an internal cavity or duct. In multicellular organisms, ducts and cavities covered by epithelia, allow the individual to maintain in a separated fashion contents so diverse as urine, blood, gastric juice, bile, milk etc. The vectorial transport of ions, water and molecules through epithelia regulates the composition of body fluids and in consequence the uptake and removal of multiple ions and substances from the body. In order to traverse an epithelium two ways are available: the transcellular and the paracellular routes. To cross through the transcellular pathway, the molecule needs to be hydrophobic to traverse the plasma membrane or alternatively to count with a specific transport mechanism such as a channel, pump, carrier or cotransporter. Passage through the paracellular route is instead regulated by a structure named the tight junction (TJ), located at the limit between the apical membrane facing the lumen and the basolateral surface in contact with the interior of the organism. TJs observed by transmission electron microscopy (TEM) appear as sites where the intercellular space between neighboring cells is obliterated and the adjoining membranes appear to fuse [1]. By freeze–fracture (FF) TJs are detected as a network of strands that encircles the cell bellow the apical surface [2].

The barrier function of TJs allows this structure to regulate by size and charge the transit of ions and molecules through the paracellular route [3]. The permeability through the paracellular pathway can be evaluated by two methods, the measurement of transepithelial electrical resistance (TER) [4] and the flux of tracers that can be detected either for being electron-dense (e.g., lanthanum, ruthenium red) and hence observable by the electron microscope or for having a fluorescent (e.g., FITC–dextran) or radioactive label (e.g., ^{3}H-mannitol). The size of the TJ pores can be evaluated employing paracellular tracers of different sizes while the ionic selectivity of the TJ can be determined measuring dilution potentials.

Besides functioning as a barrier, TJs work as a fence that limits the free movement of lipids and proteins within the plasma membrane between the apical and the basal surface [5,6]. Recently, another role for TJ has begun to be unraveled. It involves the control of cell proliferation and gene expression and requires the shuttling of TJ associated proteins from the plasma membrane to the nucleus [7].

TJs are integrated by a complex array of integral and peripheral proteins which associate to diverse molecules involved in signaling cascades, such as G proteins, kinases and phosphatases. These interactions allow the cells to transmit information on the degree of cell–cell contact to the cell interior. A significant body of evidence has accumulated in recent years indicating that the crosstalk of diverse signaling pathways regulate the formation as well as the disassembly of TJs. In this review we will focus on how several signaling pathways modulate the barrier function of the junction and how particular molecular constituents of the TJ are phosphorylated by diverse kinases. To accomplish this task we will start by providing a brief description of the main TJ proteins.

2. Brief description of the molecular constituents of the TJ

TJs are multiprotein complexes composed of integral proteins that associate with cytoplasmic plaque proteins. The former mediate cell–cell adhesion, while the latter function as a bridge between the TJ and the actin cytoskeleton (for extensive reviews, see [8,9]). Here we will only describe the main characteristics of the ZO proteins, occludin and claudins since their interaction with kinases, phosphatases and small G proteins has been more thoroughly studied.

2.1. ZO proteins of the TJ

ZO-1, ZO-2 and ZO-3 are members of the MAGUK (membrane-associated guanylate kinase) protein family and are an important subgroup of the PDZ proteins of the TJ (for reviews, see [10,8]). ZO proteins are identified for having an amino domain that contains the characteristic features of all MAGUK proteins: PDZ domains, followed by SH3 and GuK regions; and a carboxyl end that includes an acidic domain and a proline-rich region. In ZO-3 the latter is located between PDZ-2 and PDZ-3.

ZOs are peripherally associated membrane proteins whose name derives from the initials of zonula occludens, the Latin name for TJs, and whose number reflects the order in which they were originally identified (for a review on these proteins, see [10]). These proteins interact together and anchor membrane proteins like claudins, occludin and JAMs to the actin cytoskeleton. ZO proteins are scaffolds that establish numerous protein–protein interactions that cluster at the TJ diverse kinases, phosphatases, small G proteins and nuclear and transcription factors [11]. ZO-1, ZO-2 and ZO-3 contain several nuclear localization and exportation signals (NLS and NES) [11]. ZO-1 and ZO-2 concentrate at the nucleus when subjected to adverse conditions such as chemical stress or mechanical injury, or when cells are cultured at a sparse density [12–14]. ZO-1 and ZO-2 associate with proteins involved in the regulation of gene transcription and cell proliferation such as the transcription factors ZONAB [15], Jun, Fos, C/EBP [16] and KyoT2 [17], the chromatin component SAF-B involved in the assembly of transcriptosomes [14], the cell cycle regulator Cyclin D1 [18] and the heat shock protein Apg-2 [19].

2.2. Claudins

Claudins constitute a family of more than 20 proteins with four transmembrane domains [20]. They are the major component of TJ strands observed by FF. Claudins have two extracellular loops that display variability in the distribution and number of charged residues. This feature is crucial as it determines the paracellular ionic selectivity of the TJ. Accordingly, some claudins form paracellular ion channels (e.g., claudin-2, is a cation channel [21], and claudin-16 is a Mg^{2+} channel [22]) while others constitute paracellular ion barriers (e.g., claudin-8 reduces paracellular Na^{+} permeability [23]). Although claudins are found in all epithelial and endothelial cells, their tissue distribution is specific. For example, the mammalian nephron displays a wide spectrum of claudins including claudins-1, -2, -3, -4, -7, -8, -10, -11, -14 and -16 whose distribution varies in each tubular segment (e.g., the leakier segment of the nephron expresses claudin-2, whereas the tighter distal tubules exhibit claudins-7 and -8) [24,25]. Endothelial cells also display a claudin-specific distribution. Thus endothelial cells of the brain express claudins-3, -5 and -12 [26–28], kidney endothelia have claudins-5 and -15 [24,27], while placental endothelia exhibit claudins-1, -3 and -5 [29,30]. The expression of claudins also varies along the vascular tree. Thus in the placenta the expression of claudins-1 and -3 diminishes or is even absent in the smaller vessels, while the expression of claudin-5 is strong in some vessels and absent from others even of a similar width [29].

2.3. Occludin

Occludin is a TJ protein whose physiological role still remains controversial. This tetraspan protein has a first extracellular loop enriched with tyrosine and glycine residues and a second one full of tyrosines. Occludin is present in the filaments that constitute TJs [31] and its overexpression or mutations severely affect TER [32,33], yet occludin knock-out mice are viable and exhibit TJs with an apparent normal morphology [34].

3. TJs are regulated by phosphorylation

The relationship between the degree of protein phosphorylation and TJ function was first analyzed in the late 1980s by Stevenson et al. [35], demonstrating how in low resistance cells the TJ protein ZO-1 is significantly more phosphorylated than in high resistance monolayers. Since then an enormous amount of research has been done on the phosphorylation of TJ proteins. For many years, much of the results obtained remained controversial due to the fact that phosphorylation can be

achieved by the action of different kinases over distinct residues on the same TJ proteins. These results are now better understood thanks to the use of inhibitors and stimulators specific for certain kinase isoforms and to the analysis by point mutation of particular phosphorylated residues.

3.1. PKC signaling

Protein kinase C (PKC) has long been recognized to affect epithelial and endothelial barriers. PKC consists of a family of Ser/Thr-specific kinases which includes 12 known isozymes that can be classified into three subfamilies: conventional (α, β1, β2 and γ), novel (δ, ε, θ, η and μ) and atypical (λ, τ and ζ). These isoforms differ in their mechanism of action, subcellular distribution, substrate type and expression. Thus conventional isoforms (cPKC) are both Ca^{2+} and diacylglycerol (DAG) dependent, novel isoforms (nPKC) are Ca^{2+} independent but DAG dependent while the atypical PKC isoenzymes (aPKC) are neither Ca^{2+} nor DAG dependent. At the plasma membrane the nPKC isoenzymes δ and θ [36], and the atypical isoforms λ and ζ [36–42], are found associated to TJ proteins (Table 1). PKC activity can be regulated by other PKCs. Therefore a given PKC like the cPKCα may be regulating TJs even though it does not itself localize directly to the TJ.

3.1.1. PKC and TJ barrier function

The initial studies designed to elucidate the role of PKC on TJ function were performed employing stimulators and inhibitors that acted upon several subfamilies of PKC isoforms. Therefore understanding the role of specific PKC isozymes on TJs became particularly complex and required the comparison of results obtained with various PKC affecting drugs on different biological systems.

Table 1
Kinases and phosphatases present at the TJ

Kinase/ Phosphatase	Isoform	Cell or tissue	Associated to TJ protein	References
PKC	δ, θ, λ and ζ	Mouse trophoectoderm	ZO-1α+	[36]
	λ	Epidermis	Par3/Par6 complex	[37]
		MDCK and intestinal epithelia	ASIP/Par3	[38]
		MDCK	ZO-2	[39]
	ζ	MDCK, Caco-2	ZO-1	[40,41]
		MDCK	ZO-2	[39]
	ζ	T-84	Occludin	[42]
CK	Iε	HUVEC	Occludin	[260]
ERK1		Caco-2	Occludin	[160]
WNK4		Distal nephron		[229]
c-Yes		MDCK, T-84	Occludin	[269,42]
PI3K		T-84, Caco-2	Occludin	[42,209]
		Con8	ZO-1	[162]
PP2A		MDCK	ZO-1, occludin, claudin-1	[125]
		Caco-2	Occludin p-Thr residues	[126]
PP1		Caco-2	Occludin p-Ser residues	[126]

Epithelial cell lines: MDCK, dog renal; Caco-2, human colon; T-84, human colon; Con8, rat mammary tumor. Endothelial cell line: HUVEC, human umbilical cord.

The participation of PKC in TJ assembly is demonstrated by observing that (1) diC8, a DAG analogue that stimulates conventional and novel PKC, triggers the translocation of TJ proteins to the cell borders [43,44]; (2) when cells cultured in low calcium media (LC, 1–5 μM Ca^{2+}) are switched to normal calcium (NC, 1.8 mM) media, TER development can be hampered by inhibitors of conventional and novel PKC such as staurosporine, calphostin C and Gö6850, and by pan-PKC inhibitors like GF-109203X and H7 [40,44–47]; (3) EGF and fetal bovine serum (FBS) promote TJ assembly in cancerous cells in a process that can be reversed with calphostin C and Gö6850 [48]; and (4) the bacteria *Helicobacter pylori* disrupts the barrier function of intestinal epithelial cells through a process inhibited by PMA an activator of novel and conventional PKCs [49].

The participation of PKCs on TJ disassembly has been studied employing diverse protocols that trigger TJ disruption such as oxidative stress [50]; removal of Ca^{2+} from the media [39,44,51–53]; elevation of intracellular Ca^{2+} by treatment with the Ca^{2+} ionophore A23187 [54], incubation with different agents such as phorbol esters [55,56]; hepatocyte growth factor (HGF) [57] and vascular endothelial growth factor (VEGF) [58]; the hormone vasopressin [59]; cytokines tumor necrosis factor α (TNFα) and interferon γ (IFNγ) [60]; HIV-1 envelope glycoprotein gp120 [61]; toxins Zot (zonula occludens toxin), from *Vibrio cholera* [62] and A from *Clostridium difficile* [63], and incubation with OmpA (outer membrane protein A)-positive *Escherichia coli* [64] or enterohemorrhagic *E. coli* (EHEC) [65]. These studies have revealed that inhibitors of classical and novel PKCs like staurosporine, Gö6850 and CGP41251, as well as the pan-PKC inhibitors GF-109203X and H7 attenuate the opening of TJs. Interestingly some of these studies have further shown that TJ opening is accompanied by the translocation of cPKCα from the cytoplasm to the plasma membrane [66–69,58,64,62]. In fact, chronic exposure of epithelial LLC-PK1 cells to phorbol esters results in a partial TER recovery and a reciprocal change in mannitol permeability. These effects triggered by the chronic exposure to phorbol esters are due to the downregulation of cPKCα [68,69].

The effect of phorbol esters that stimulate both cPKC and nPKC varies on different biological systems. Thus, phorbol esters improve the TJ barrier function in colon carcinoma (HT-29) cells [70] and in a certain clone of pig renal cells (LLC-PK1A) [71]. In mouse pre-implantation embryo phorbol esters stimulates membrane assembly of ZO-1 isoform α+ [72]. Instead the acute treatment with phorbol esters induces in diverse epithelial cell lines like LLC-PK1 (renal), MDCK (renal), T-84 (intestine) and HepG2 (hepatic) a decrease in the barrier function of the TJ manifested by a drop in the TER, an increased paracellular permeability, the disappearance of TJ proteins from the cell borders, as well as the appearance of a disorganized pattern of TJ strands [54,56–58,66,67,71,73–75]. In LLC-PK1 cultures chronic treatment with phorbol esters triggers the abovementioned recovery of TER as well as the appearance of multilayered polyp-like foci (PLFs), across the otherwise one cell layer thick cell sheets. These PLFs have TJs permeable to ruthenium red and exhibit cPKCα in the membrane-associated

compartment, in contrast to the adjacent morphologically normal epithelium that has sealed TJs and no discernible cPKCα expression [68,69]. In the choroid plexus epithelium the alteration of TJs induced by phorbol esters can be blocked with Rottlerin, a specific inhibitor of nPKCδ, thus suggesting participation of this isozyme in TJ disassembly [76].

The role played by PKCδ and α on TJs is complex and seems to vary according to the cellular context. For example, the overexpression nPKCδ in renal LLC-PK1 cells exerts a negative effect on the TJ barrier [73]. In contrast rottlerin blocks in MDCK cells the formation of the TJ barrier triggered by Ca^{2+} [44], and in the intestinal epithelial cells HT-29 and Caco-2, the stimulation of Toll-like receptors 2 (TLR2) with specific ligands leads to the activation of the cPKCα and the nPKCδ, the development of an enhanced TER and the translocation of ZO-1 to the TJ [77]. These effects could be blocked with rottlerin and the cPKC inhibitor Gö6976. Taken together these results indicate that in some tissues like the choroid plexus epithelium and LLC-PK1 cells, nPKCδ and cPKCα promote TJ degradation while in others such as HT-29, Caco-2 and MDCK these isozymes favor TJ formation (Fig. 1).

The importance on TJ formation of novel and atypical PKC isoforms δ and ζ has been highlighted in studies done in mouse pre-implantation embryos where these isozymes concentrate at the TJ [36]. In immunosurgical isolated inner cell masses (ICM), membrane assembly of ZO-1α+ and ZO-2 triggered by indolactam, an alkaloid activator of cPKC and nPKC isoenzymes, and of ZO-1α+ elicited by TPA, can be blocked with the pan-PKC inhibitor Gö6983, and the conventional and novel inhibitors Ro-31-8220 and Ro-31-8425, but not by Gö6976, an inhibitor of cPKC isoforms [72]. These results thus suggest that nPKCδ and aPKC but not cPKCs, play a central role in TJ formation (Fig. 1). This proposal was further confirmed when specific inhibitors of nPKCδ (translocation inhibitor peptide) and aPKCζ (pseudosubstrate peptide) blocked the assembly of ZO-2, and inhibiting the activity of aPKCζ attenuated ZO-1α+ assembly [78]. In contrast to these observations, *E. coli* Nissle 1917 (EcN), which has been employed for decades for the treatment and prevention of intestinal disorders in Central Europe, restores the epithelial barrier disruption generated by enteropathogenic *E. coli* (EPEC) strain, by silencing the aPKCζ and triggering the enhanced expression of ZO-2 and its redistribution towards the cell borders [79].

nPKCθ also appears to be crucial for TJ stability as its under or overexpression in Caco-2 cells reduces the monolayer barrier function, the presence of claudins-1 and -4 at the plasma membrane as well as their degree of serine and threonine phosphorylation [80].

Bryostatin 1, a non-phorbol ester stimulator of cPKCα, and the nPKCε and δ, is a relatively novel pharmacological tool to uncover the role that different PKC isoforms play in junction physiology. Thus in LLC-PK1 epithelial cells, bryostatin 1 causes only a 30% decrease in TER which spontaneously reverses after 5 h. This effect can be explained by the fact that in contrast to TPA, bryostatin 1 triggers a slower translocation of cPKCα from the cytosol to the membrane and a much more rapid downregulation of cPKCα, with disappearance from this compartment after only 6 h [67]. Moreover, in the human intestinal cell line T-84, bryostatin 1 increases TER, shifts the subcellular distribution of claudin-1 and ZO-2 from a detergent soluble to an insoluble fraction and triggers the appearance of phosphorylated occludin [81]. Since bryostatin 1 induces rapid and sustained activation of the δ and ε nPKC isozymes in T-84 cells after 30 min and only activates cPKCα after 3–4 h followed by downregulation [82], it has been proposed that the activation of either nPKC δ or ε triggers the increased TER. However, since bryostatin 1 effect can be attenuated with the novel and cPKC inhibitor Gö6850, but not with the cPKC

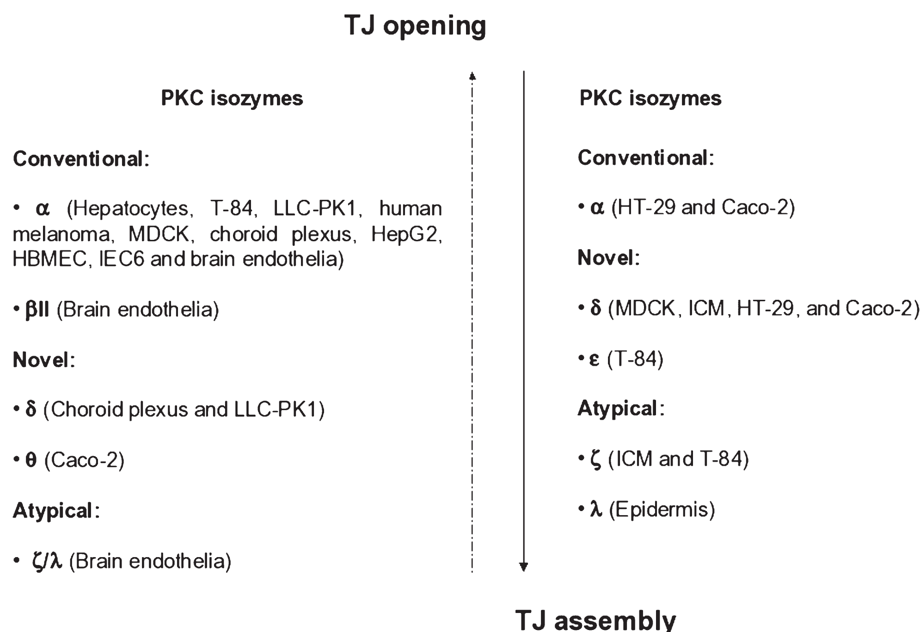

Fig. 1. Different PKC isozymes are involved in TJ opening and assembly. The name of the PKC isozyme involved and the cell line or tissue where the kinase effect was tested is indicated.

inhibitor Gö6976 or the nPKCδ inhibitor rottlerin, it has been concluded that nPKCε signaling promotes the barrier function of TJs [81].

Another evidence that suggests the participation of different PKC families on TJ formation and disruption arose with the observation that in MDCK cells the phosphorylation of occludin and the appearance of TJ proteins at the cell borders triggered by diC8 are potentiated by Gö6976, an inhibitor of cPKCs, and blocked by rottlerin, an inhibitor of the nPKCδ.

In summary we can conclude that in general cPKC participate in junctional disassembly while novel isoforms regulate junction formation [44]. However, since contradictory effects are found in different cell models, both cultured cell lines and tissue, in Fig. 1 we provide a scheme that summarizes which PKC isozyme promotes TJ opening or assembly in each particular cell system.

3.1.2. PKC and the development of cell polarity

Apicobasal polarity is a crucial characteristic of epithelia that allows the vectorial transport of ions and molecules between the external media and the interior of the organism. Three molecular complexes are responsible for cell polarity: Par3/Par6/aPKC, Crumbs/Pals/PATJ and Scribble/Disc Large/Lethal Giant Larvae (Lgl). Here we will concentrate on the first as we are particularly interested in analyzing the impact of kinases on the regulation of TJ formation, and since Par6 and aPKC regulate the other two polarity complexes [83–85].

Par3/Par6/aPKC is an evolutionary conserved complex crucial for anterior–posterior polarity in *Caenorhabditis elegans* and the apical–basal polarity of epithelial cells and neuroblasts in *Drosophila melanogaster* [86,87]. In mammalian epithelial cells, this complex is found at the apical end of the junctional complex where TJs are located. Introduction of a kinase defective aPKC mutant blocks the contact induced formation of junctional structures and inhibits the development of cell surface polarity [88,89]. Par3 exhibits an aPKC binding site, and a PKC phosphorylation site (Ser827) that becomes phosphorylated presumably by the aPKC during the initial phase of TJ formation [90]. Par3 associates directly with the TJ protein JAM and this interaction appears to tether the Par3/Par6/aPKC complex to TJs [91] (Fig. 2).

Par6 is a scaffolding protein whose single PDZ domain associates with the N-terminal PDZ of Par3, a protein containing 3 PDZ domains. At the amino terminal portion of Par6, a PB1 (Phox and Bem1p) region is located that heterodimerizes with a PB1 domain present in aPKC. A CRIB motif present in Par6, downstream of the PB1 domain, associates with active Rho proteins. This interaction enhances the kinase activity of the aPKC [89]. ECT2, a guanine nucleotide exchange factor, interacts directly with Par6 and its overexpression stimulates PKCζ activity [92]. In confluent MDCK cells ECT2 is detected in cell–cell junctions and when cells are grown in LC medium, a condition where TJs and adherens junctions are lost, the amount of ECT2 decreases and the protein concentrates at the nucleus instead of the cell borders [92]. The importance of ECT2 in cell polarization is highlighted by observing that dominant negative or constitutively active forms of ECT2 inhibit normal cyst formation in MDCK cells cultured in three-dimensional collagen gels, due to a blockade in the selective elimination of cells at the center of cysts [93].

A crosstalk between the three polarity complexes has been detected. This situation is particularly interesting since the tumor suppressor Lgl concentrates in polarized epithelial cells, at the basolateral membrane domain, whereas the Par3/Par6/aPKC complex localizes at the TJ region. The key for understanding the interaction of these physically separated complexes lies in observing that the PDZ domain of Par6 selectively interacts with either Lgl or Par3 [84] (Fig. 2). Thus initial cell contacts recruit aPKCλ/Par6/Lgl complexes to the cell–cell contact region. Interaction of Cdc42-GTP with Par6 activates the aPKCλ, triggering the phosphorylation and subsequent segregation of Lgl from the Par6/aPKCλ complex. In consequence Lgl remains confined to the lateral membrane region and the formation of the Par3/Par6/aPKCλ complex that induces TJ formation is promoted.

aPKC and Lgl appear to act antagonistically in the regulation of the apicobasal polarity in early vertebrate development [94]. Thus, in frog blastomeres the overexpression of aPKC expands the apical domain at the expense of the basolateral while the loss of aPKC function or the increased expression of Lgl causes expansion of the basolateral domain. When aPKC is overexpressed, the TJ is repositioned to the new apico-basolateral interface while it is lost when the aPKC is non-functional. The overexpression of Crumbs, a target of aPKC, causes apicalisation similar to that observed with aPKC.

The establishment of the Crumbs/Pals/PATJ complex is affected by the Par6/aPKC complex since the association of Cdc42-GTP to Par6 regulates the interaction of the amino terminus of Pals1 with the PDZ domain of Par6 [85]. This association affects the formation of the Crumbs/Pals/PATJ complex, as it competes PATJ binding to Pals1. Furthermore, aPKC directly associates to both Crumbs and PATJ, and the former is a phosphorylation target of aPKC. This phosphorylation is required for the development of epithelial cell polarity in *Drosophila* [95].

In endothelial cells, Par3 and Par6 form a complex with endothelial cadherin (VE-cadherin) that lacks aPKC. Yet this complex localizes to adherens junctions and promotes cellular polarization [96].

3.2. PKA and PKG signaling to TJs

Signaling by protein kinase A (PKA) has long been known to regulate both the assembly and opening of the paracellular route in epithelial and endothelial cells. PKA activity is promoted by treatment with permeable cAMP (e.g., dibutyryl cAMP) or with drugs that activate the enzyme adenylyl cyclase (e.g., forskolin), or that inhibit the phosphodiesterase (e.g., xanthines), and in consequence raise the intracellular level of cAMP.

PKA activation as well as phosphomimetic mutations done in PKA targets sites of TJ proteins block the development of the TJ barrier during a Ca^{2+} switch in MDCK and human ovary cells [45,97], lower the TER of gallbladder, Sertoli and MDCK cells [98–100] and induce size selective loosening in

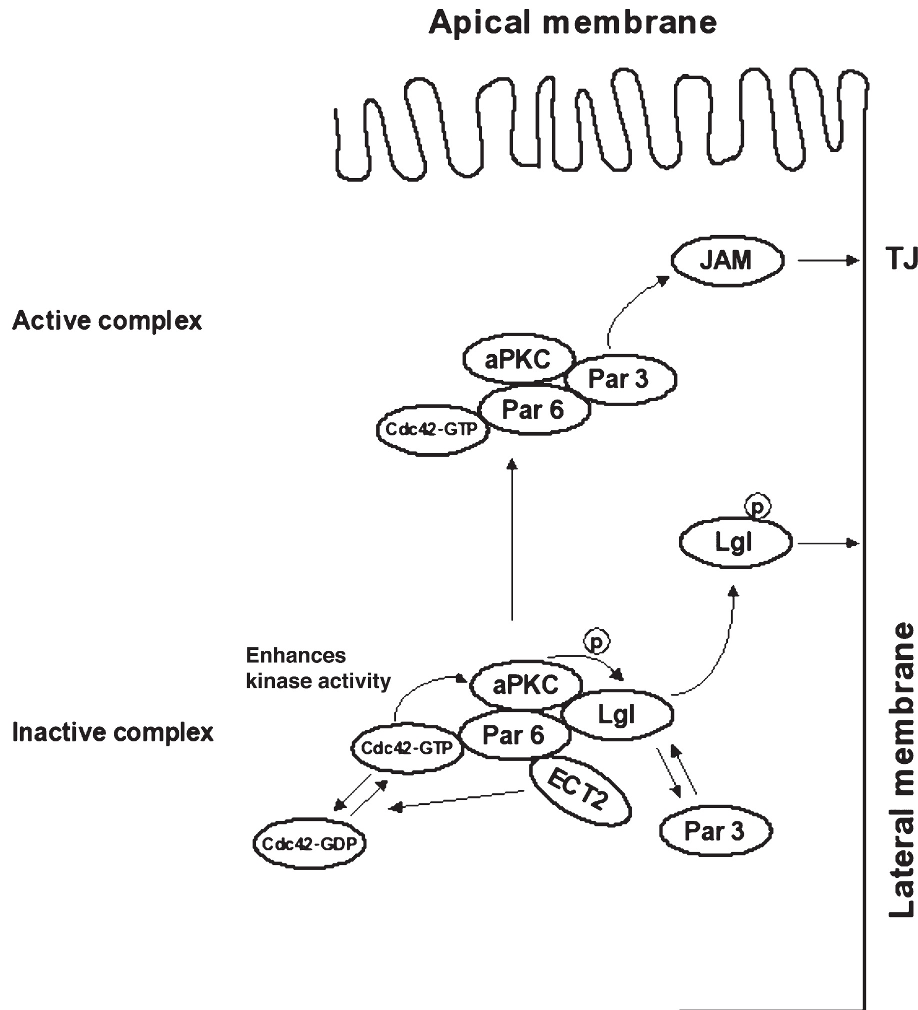

Fig. 2. Schematic model of interaction of aPKC with protein complexes involved in the genesis of cell polarity in epithelial cells. Upon establishment of the initial cell–cell contacts activated Cdc42 enhances aPKC activity, phosphorylating Lgl. This phosphorylation segregates Lgl from the Par6/aPKC complex promoting the formation of a new complex integrated by Par6/aPKC/Par3 that binds to the TJ protein JAM and promotes TJ formation. Segregated Lgl remains in the lateral membrane promoting the growth of this membrane domain.

lung endothelia [101]. Accordingly PKA inhibitors H-89 and HA-1104 preserve the TJ barrier of mammary epithelial cells [102] and block the hyperphosphorylation of ZO-2 in MDCK cells [39], upon removal of Ca^{2+} from the extracellular medium.

In the BBB astrocytes surround endothelial cells providing them with a biochemical support. Culture models of endothelial brain cells have a low TER of 10–120 Ω cm^2 and form TJ networks of low complexity [103]. TER doubles when the cells are cultured with astrocyte conditioned media or with astrocytes, but even a greater effect is obtained by increasing intracellular cAMP, reaching TER values as high as 600 Ω cm^2 [104,105]. cAMP stabilizes TJs, decreases permeability and increases the complexity of junctional strands in a rapid and reversible manner [104–107]. The addition of astrocyte conditioned medium together with cAMP changes the association of brain endothelial junctional particles, from the E to the P face of the plasma membrane, resembling the association pattern observed in epithelial TJs [107,108].

In certain epithelia like thyrocytes, hepatocytes and in the colon epithelial cell line T-84, PKA activation promotes the re-establishment of the TJ barrier triggered by a Ca^{2+} switch [109] and reverses TJ disruption generated by oxidative stress [50] and extracellular Ca^{2+} removal [110]. In invertebrates a somewhat similar effect is detected. Thus in the early and mid-vitellogenic follicles of the insect *Hyalophora cecropia*, treatment with cAMP transforms the loosely organized follicle cells that allow the intercellular movement of vitellogenin to the oocyte surface, into a tight epithelium [111].

Rab 13 works as a link between PKA signaling and TJ assembly [112]. This GTPase is recruited to cell–cell contacts at an early stage of TJ assembly, binds to PKA, inhibits its activity and delays the arrival of claudin-1 and ZO-1 to the TJ.

In summary we can conclude that in brain endothelial cells PKA activation promotes the barrier function of TJs, while in other endothelia and in epithelial cells a generalization cannot be made, as PKA can produce contrary actions in different cell models.

In brain endothelial cells activation of PKG by cGMP exerts different effects depending on the state of the tissue. Thus protein kinase G (PKG) participates in the signaling cascades that leads to opening of the BBB triggered by hypoxia [113]. Hypoxia induced hyperpermeability is mediated by the interaction of VEGF with its tyrosine kinase receptors (e.g., VEGF-R2) [114]. This leads to receptor dimerization followed by autophosphorylation, which in turn triggers the activation PI3K/AKT and PLCγ. The latter induces generation of IP3 that produces an elevation in intracellular Ca^{2+} that activates nitric oxide synthase (NOS) to produce NO [115–117]. NO stimulates soluble guanylate cyclase (GC) leading to increase cGMP, which in turn activates PKG. Inhibitors of eNOS (e.g., NG-monomethyl-L-arginine), GC (ODQ) and PKG (KT 5823) abolish hypoxia and VEGF induced permeability changes [114,117,118] as well as the delocalization of ZO-1 and ZO-2 from the cell borders [113], thus demonstrating that both GC and PKG are involved in TJ opening in brain endothelial cells triggered by hypoxia. Hypothermia can decrease hypoxia induced hyper permeability by lowering the expression of VEGF and with it the levels of NO and cGMP [119].

In contrast, cGMP and PKG have also been reported to increase the barrier function of brain endothelial cells. Thus NO donors (e.g., SNP and DETA NONOate) or cGMP agonists (8-Br cGMP) increase TER, decrease paracellular permeability, and are able to reverse the increased permeability triggered by cytokines in HBMEC cultures [120], or by bradykinin infused into the carotid artery of rats implanted with gliomas [121]. Accordingly inhibiting cGMP (zaniprast) enhances the effect of drugs like Cereport, a bradykinin agonist, that increase permeability of the brain microvascular endothelium [122].

The importance of NO signaling in junctional sealing is further highlighted in the microgravity condition. Thus bovine aortic endothelial cells (BAEC) and liver WB-F344 epithelial cells, cultured in rotary wall vessels to stimulate a microgravity environment, form large cellular aggregates that display an increase in barrier properties and upregulation of TJ protein expression, through a process that involves increased basal production of NO [123,124].

In summary the discrepancy between the results that show increase TJ sealing by the NO signaling pathway and those obtained in hypoxia could be due to the fact that in the latter condition the effect of cGPM and PKG is given under a more complex background that involves the simultaneous activation of several other second messengers.

3.3. TJs and phosphatases PP1, PP2A and PP2B

Ser/Thr phosphorylation of several TJ proteins promotes the barrier function of TJs. Therefore it is expected that reducing the phosphorylation level of such proteins would negatively affect TJ integrity. Such appears to be the case since enhancing the activity of the Ser/Thr protein phosphatase 2A (PP2A) induces dephosphorylation of ZO-1, occludin, and claudin-1, and an increased paracellular permeability, while inhibition of PP2A with okadaic acid promotes the phosphorylation of these TJ proteins and accelerates junctional assembly [125,126].

Protein phosphatases PP2A and PP1 interact with the carboxyl terminal tail of occludin (Table 1). PP2A dephosphorylates occludin on Thr, while PP1 dephosphorylates Ser residues. Na^{+},K^{+}-ATPase in mammalian cells localizes at TJs and associates with PP2A. Inhibition of Na^{+},K^{+}-ATPase ion transport function reduces PP2A activity and hyperphosphorylates occludin. However, in pancreatic cells, inhibition of PP2A with ouabain decreases TER and increases paracellular permeability [127]. Altogether these results suggest that altering the balance of occludin phosphorylation in Ser/Thr residues exerts a profound effect on the gate function of TJs.

In the relationship between phosphatases and TJ proteins, PKC is also involved. PP2A associates with aPKCζ, an isoform found at TJs. This interaction inhibits the activity of the kinase and promotes its relocalization to the cytosol. Moreover, the inhibition of aPKCζ can block the appearance of TJ proteins at the cellular borders induced by okadaic acid [125]. PP2B in turn appears to decrease the phosphorylation state of cPKCα, and treatment with PP2B inhibitor FK506 blocks the recovery of the thrombin-mediated decrease of TER in pulmonary endothelial cells [128]. This result is in agreement with previous observations demonstrating that cPKCα promotes TJ disassembly [44]. Thrombin-induced TJ barrier disfunction in endothelial cells is mediated by an increased phosphorylation of MLC that triggers contraction of the acto-myosin ring. This process appears to be determined by an increased activity of PP2B triggered by thrombin, as PP2B inhibitors (e.g., cyclosporin and deltamethrin) attenuate MLC dephosphorylation [129].

In summary we conclude that Ser/Thr phosphatases exert opposite effects on the barrier function of TJs by inhibiting different PKC isoform. Thus PP2A inhibits the aPKCζ involved in TJ assembly and hence this phosphatase triggers TJ opening, whereas PP2B blocks the activity of cPKCα that disassembles TJs and in consequence PP2B promotes TJ sealing.

3.4. Rho signaling and TJ function

Rho is a member of the Ras superfamily of small GTP binding proteins that switch between active GTP bound and inactive GDP bound conformations. This process is regulated by GDP to GTP nucleotide exchange factors and by GTP hydrolysis stimulated by GTPase activation proteins. Rho family of GTPases includes RhoA, Rac and Cdc42 that regulate distinct actin structures in response to diverse signals. The role of Rho signaling in TJ function has been studied employing constitutively active mutants, that have a low basal rate of GTP hydrolysis, and are thereby locked in an active state, and dominant negative mutants that have a preferential affinity for GDP. Studies employing both dominant active and negative mutants of RhoA, Rac and Cdc42 reveal that they all disrupt the barrier function of TJs, yet the most intense effect is obtained with dominant active RhoA mutants [130,131].

C3 transferase from *Clostridium botulinum*, ADP ribosylates RhoA, but not Rac and Cdc42, and therefore has been employed to specifically inactivate RhoA. Downstream effectors of RhoA include the family of Ser/Thr kinases termed p160 ROCK/ROKb/ROCKI and Rho kinase/ROKa/ROCKII. ROCKs can

induce acto-myosin contractility by a direct phosphorylation of the regulatory myosin light chain (MLC2) or the myosin phosphatase target subunit (MYPT) resulting in the inhibition of myosin phosphatase and hence hyperphosphorylation of MLC2 (Fig. 3). Several plaque proteins of the TJ interact directly with actin (e.g., ZO-1, ZO-2, ZO-3, AF-6 and cingulin) and myosin (e.g., cingulin). Such interactions stabilize the junctional complex at the cell border and also provide the force for the disruption of intercellular junctions upon contraction of the perijunctional acto-myosin ring. Therefore it is no surprise that the RhoA/ROCK signaling pathway participates both in the assembly and disassembly of TJs. Hence during ATP depletion activated RhoA better maintains junctions, while RhoA inhibition results in a more extensive loss of junctional components in MDCK cells [132]. Moreover, RhoA inactivation with C3 transferase disrupts TJ structure and function in Caco-2, T-84 and MDCK cells [133,134]. In the latter it inhibits both TJ and adherens junction assembly [135]. Direct inhibition of ROCK with Y27632 disorganizes the apical ring of actin but does not alter TJ protein localization. However, if ROCK is inhibited during TJ assembly triggered by a Ca^{2+} switch, TJ proteins fail to redistribute at the intercellular junctions, demonstrating that ROCK is critical for the assembly of TJ proteins [134].

TJ opening by activation of the RhoA signaling pathway is triggered by several factors including pro-inflammatory cytokines in intestinal T-84 cells [136], microtubule destabilizing drugs in human pulmonary artery endothelial cells [137,138] and the migration of small lung cancer cells through brain endothelial cells [139]. The expression of pro-inflammatory cytokines like IFNγ is a hallmark characteristic in the pathogenesis of inflammatory bowel diseases such as Crohn's and ulcerative colitis, while the microtubule destabilizing drug 2 methoxyestradiol (2ME), a derivative of the main natural estrogen 17β estradiol, is currently being tested in the therapy for several cancers. Therefore understanding the mechanism of action of these molecules upon epithelial barriers has enormous clinical importance. Both IFNγ and 2ME activate RhoA,

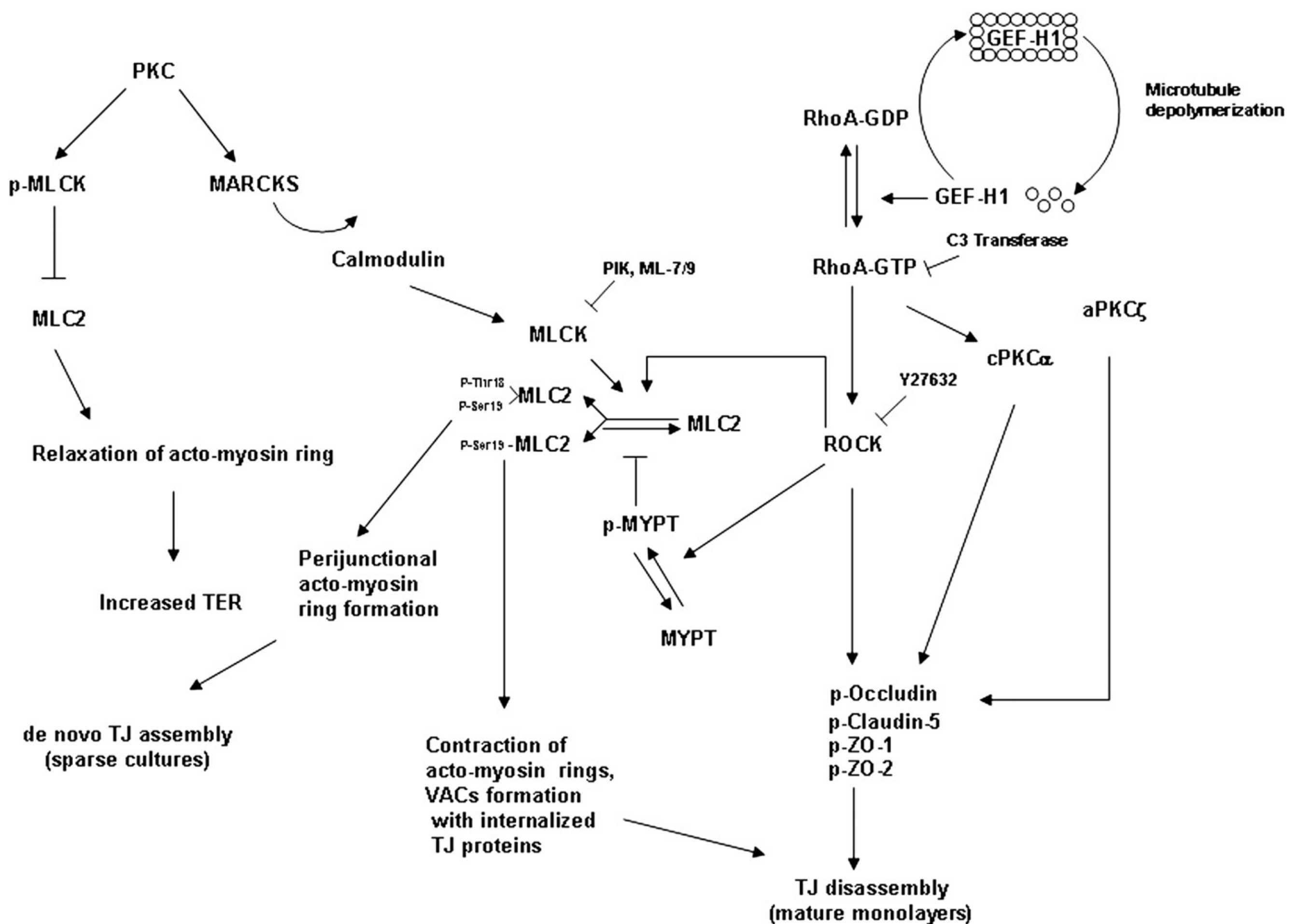

Fig. 3. The crosstalk between PKC, Rho and MLCK signaling pathways regulates TJs. Microtubule depolymerization allows GEF-H1 to stimulate the exchange of GDP to GTP on Rho. Activated RhoA signals on its downstream target ROCK resulting in phosphorylation of TJ proteins, MLC2 and MYPT. In brain endothelial cells, BBB impairment is accompanied by Ser/Thr phosphorylation of occludin, claudin-5, ZO-1 and ZO-2, activation of cPKCα and aPKCζ and Rho/ROCK signaling. Phosphorylation of MLC2 triggers contraction of acto-myosin rings and TJ disassembly in certain epithelia like MDCK, Caco-2 and T-84. Phosphorylation of MLCK by PKC inhibits the activity of the former, leading to a decrease phosphorylation of MLC2, relaxation of perijunctional acto-myosin and increased TER in some epithelia including Caco-2 cells. PKC phosphorylation of MARCKS releases calmodulin available for MLCK activation. Phosphorylation of MLC2 triggers in T-84 cells the formation of acto-myosin rings that favor de novo junction formation in sparse cultures and in assembly assays such as the Ca^{2+} switch.

triggering the subsequent upregulation of ROCK, the phosphorylation of MLC2 via a MLCK-independent pathway, TJ disassembly and increased paracellular permeability. In the case of IFNγ the formation of large actin coated vacuoles (VAC) that originate from the apical membrane and internalize TJ proteins occludin, JAM-A and claudin-1 has further been observed [136]. Employment of ROCK siRNAs has served to distinguish the participation of ROCK2 and not ROCK1 in junctional disruption triggered by microtubule destabilization with 2ME [137].

The link between microtubule destabilization and RhoA activation resides in the nucleotide exchange factor GEF-H1 [140]. This factor that activates RhoA but not Rac or Cdc42 associates with microtubules, and such association inhibits its activity. Upon microtubule depolymerization by nocodazol or thrombin treatment, GEF-H1 becomes active stimulating in consequence RhoA signaling [138,140]. During cytokinesis the depolymerization of spindle microtubules could speculatively trigger GEF-H1 activation and the subsequent RhoA/ROCK signaling, that would then be expected to promote the assembly of intercellular junctions between the two daughter cells. Instead in treatments that promote TJ opening by microtubule destabilization, GEF-H1 stimulation of RhoA is expected to induce the contraction of acto-myosin rings and the subsequent endocytosis of TJ components.

GEF-H1 interacts at the TJ with cingulin a plaque protein of the TJ [141,142]. This interaction inhibits RhoA activation. In sparse cultures where the expression of cingulin is low, GEF-H1 is cytoplasmic and RhoA is active. Instead, in high density cultures GEF-H1 is sequestered by cingulin at TJs and RhoA activation is inhibited.

In summary the abovementioned results suggest that RhoA activation is important for de novo formation of TJs occurring in sparse cultures and in monolayers subjected to a Ca^{2+} switch assay. The cellular level of activated RhoA is critical for TJ physiology, therefore overthrowing this balance with pro-inflammatory cytokines like IFNγ or with microtubule destabilizing drugs, as well as the inactivation of RhoA with C3 leads to TJ disassembly (Fig. 3).

3.5. Myosin light chain kinase and TJs

The motor protein myosin II is composed of two heavy chains of 200 kDa each and four light chains of 20 kDa, two each of so-called essential and regulatory light chains (MLC1 and MLC2, respectively) (Fig. 4). The heavy chains form a parallel two chain structure with a coiled-coil region termed rod or tail from which a pair of large globular NH_2 terminal heads project. The heads interact with ATP and actin to generate force and filament sliding.

The mechanism by which epithelial TJs are disassembled involves the phosphorylation at serine 19 of MLC2, that leads to the contraction of the acto-myosin belt. Reducing the extracellular calcium concentration to the micromolar range provokes the disassembly of tight and adherens junctions through a process that triggers the centripetal retraction of perijunctional actin and myosin filaments and the formation of contractile acto-myosin rings where internalized AJ and TJ proteins are co-localized [143,144]. This process can be hampered by actin stabilization (jasplakinolide) or depolymerization (cytochalasin D or latrunculin A), blocking myosin in an actin detached state (blebbistatin) or by inhibiting MLCK (ML7). The importance of MLCK in TJ disassembly is further reinforced by observing that (1) the overexpression of MLCK catalytic domain increases the phosphorylation of MLC2, reorganizes perijunctional actin, alters the distribution of TJ proteins and increases the paracellular permeability in epithelial cells [145,146] and (2) the increased paracellular permeability of epithelial cells triggered by transfection with the intestinal Na^+–glucose cotransporter SGLT1 [147], incubation with HIV-1 envelope glycoprotein gp120 [61], infection with EPEC or EHEC [148,65], treatment with TNFα and IFNγ [148] or bile acids [149] is mediated by MLC2 phosphorylation by MLCK, as this process is blocked by MLCK inhibitors ML7, ML9 and PIK. Interestingly the incubation with the yeast *Saccharomyces boulardii* that reverses the increased intestinal permeability triggered by EHEC abolishes EHEC induced MLC phosphorylation [150].

The assembly of the apical junctional complex is also dependent on the phosphorylation of MLC2 and requires the

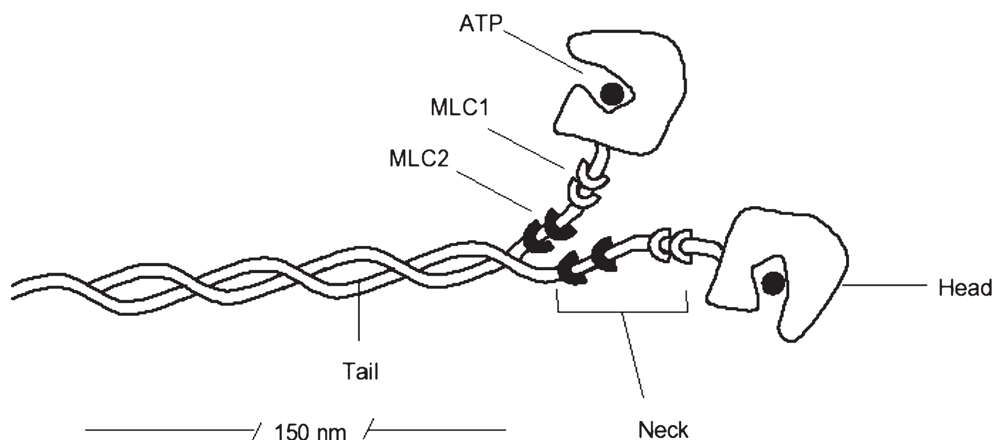

Fig. 4. Model of myosin II molecule. Myosin II is composed of two heavy chains that form a parallel structure organized as a coiled-coil rod from which large globular heads project. Two light chains are found around each neck: an essential light chain (MLC1) and a regulatory light chain (MLC2).

participation of polymerized actin [151]. Thus during a Ca^{2+} switch assay TJ proteins appear at the cellular borders in co-localization with actin, actin microfilament nucleation proteins Arp2/3 and N-WASP (neuronal Wiskott–Aldrich syndrome protein), myosin II and diphosphorylated MLC2 at Thr18/Ser19. The amount of the latter increases significantly during the Ca^{2+} switch assay. The arrival of TJ proteins to the cell borders triggered by Ca^{2+} is blocked by (1) actin depolymerization with cytochalasin D or latrunculin A; (2) the N-WASP inhibitor, wiskostatin and (3) the myosin blocker blebbistatin, which also inhibits the ability of epithelial cells to develop a columnar phenotype with a proper apico-basal polarity.

In summary these results suggest that TJ opening triggered by incubating cells in LC media, treatment with cytokines, viral proteins or bacterial infections involves the phosphorylation of MLC2 in Ser19, that leads to the contraction of the actomyosin ring, while the diphosphorylation of MLC2 at Thr18/Ser19 triggers de novo assembly of the junctional complex during a Ca^{2+} switch assay (Fig. 3). Both processes depend on an intact cytoskeleton of actin and myosin.

A crosstalk between PKC and MLCK appears to be involved in the regulation of TER (Fig. 3). Thus, in intestinal cells PKC activation is accompanied by a progressive decrease in MLC2 phosphorylation. Interestingly an acute increase in MLCK phosphorylation that occurs concurrently with PKC activation precedes the decrease in MLC2 phosphorylation and increased TER [152]. Hence a model has been proposed in which PKC activation triggers the phosphorylation of MLCK. MLCK activity is inhibited by intramolecular interactions between its catalytic domain and the calmodulin binding inhibitory domain. In the presence of Ca^{2+} and calmodulin, this intramolecular interaction is blocked, the inhibition is released and the enzyme is activated. However, if PKC and PKA target sites present in the calmodulin binding domain of MLCK are phosphorylated, MLCK activity is inhibited due to interference with calmodulin-dependent enzyme activation. Therefore MLCK inactivation due to phosphorylation by PKC leads to decreased MLC2 phosphorylation, relaxation of the perijunctional acto-myosin ring and increased TER. In contrast, infection of T-84 cells with EHEC and treatment of brain microvascular endothelia with gp120 from HIV-1 induce an opening of TJs through a process that involves the activation of both PKC and MLCK. These two signaling molecules could cause the same patho-physiological outcome acting at different sites within the cytosol, or alternatively PKC could be regulating MLCK activity by modulating the availability of calmodulin. In this respect it is important to note that PKC phosphorylation of MARCKs (myristoylated alanine-rich C kinase substrate) releases calmodulin bound to MARCKs that is then available for MLCK activation [153].

3.6. MAP signaling and TJs

Mitogen-activated protein (MAP) kinases are Ser/Thr protein kinases that respond to extracellular stimuli such as growth factors and stress, and regulate various cellular activities including gene expression, mitosis, differentiation and apoptosis. This signaling pathway has a membranous and a cytoplasmic phase. The first occurs in close proximity to the growth factor receptors and involves the activation of small GTP binding proteins (i.e., Ras). This is followed by the sequential activation of several cytoplasmic kinases known as MAPK. MAP3K is the first kinase in the cascade to become activated, and as a result phosphorylates a MAP2K on Ser/Thr residues, and then this MAP2K activates a MAPK by phosphorylation on its serine and tyrosine residues. MAPK can phosphorylate a large number of proteins that are located both in the cytoplasm and the nucleus, including transcription factors that regulate the transcription of diverse genes.

Four distinct groups of MAPKs are present in mammals: the first which is activated by growth factors and regulates cell proliferation and differentiation is integrated by the extracellular signal-regulated kinases (ERKs) also known as classical MAP kinases. ERK1 has been found in epithelial cells to interact with occludin (Table 1). Other two groups of MAPKs activated by stress stimuli are c-Jun N-terminal kinases (JNKs) and p38 isoforms, which are involved in cell differentiation and apoptosis. A fourth group named ERK5 has been found to be activated by both growth factors and stress stimuli and is known to participate in cell proliferation.

MAP signaling pathway is able to modulate TJ paracellular transport by up- or downregulating the expression of several TJ proteins (Tables 2 and 3) and hence altering the molecular composition within TJ complexes. For example, the activation of ERK1/2 by transfection with an activated Ras mutant increases sixfold the transepithelial permeability of mannitol and more than 40% the TER. This decreased paracellular permeability to NaCl and increased permeability to a nonelectrolyte is not due to loss of junctions as there is a ceiling to the size of solutes that can cross the barrier (10,000 Da methylated dextran can cross while 2,000,000 Da methylated dextran cannot), and instead occurs in response to a decline to unde-

Table 2
Studies where MAPK signaling cascade favors the barrier function of TJs

Stimulus	MAPK involved	Altered TJ protein	Cell type	References
TGFβ	ERK1/2	↑ Cl-1 and ZO-2	MDCK, T-84	[158,159]
EGF	ERK1/2	ND	Caco-2	[160]
HGF	ERK1/2	↓ Cl-2	MDCK-II, 94D	[155]
IL-17	ERK1/2	↑ Cl-1 and Cl-2	T-84	[157]
Endostatin	ERK1/2	↑ Occludin	Retinal vessels	[163]
Protectants of oxidative stress (thiol)	ERK1/2, JNK, p38	ND	Lung endothelia	[270]
Glucocorticoids	ERK1/2	None	Con8	[162]
Steroids: estradiol and dihydrotestosterone	ERK1/2	↑ Occludin	HUVEC	[164,165]
Bile	ERK1/2	↑ Occludin and ZO-1	Ileal mucosa	[161]

Epithelial cell lines: MDCK, dog kidney; T-84, human colon; Caco-2, human colon; 94D, mouse cortical collecting duct; Con8, rat mammary tumor; endothelial cell lines: HUVEC, human umbilical cord. ↑ Increased or ↓ decreased expression of indicated TJ protein. Cl, claudin.

Table 3
MAPK signaling cascade perturbs the barrier function of TJs

Stimulus	Activated MAPK	Altered TJ protein	Cell type	References
Ras transfection	ERK1/2	Occludin, Cl-1 and ZO-1 disappear from cell borders	MDCK	[166,154]
		↓ Cl-2 and ↑ occludin, claudins 1, 7 and 7	LLC-PK1	
Raf transfection	ERK1/2, p38	↓ Cl-2, altered distribution of ZO-1 at cell borders	Hepatic	[167]
Bile (cholic and taurodeoxycholic acids)	ERK1/2, p38		Caco-2, pancreatic duct epithelia	[149,169,170]
Oxidative stress (H_20_2, DMNQ)	ERK1/2	↓ Occludin, ZO-1 and ZO-2 disappear from cell borders, and ↑ P-Ser of occludin	Brain endothelia and HUVEC	[172,171,173]
Alcohol	ERK1/2, JNK, p38	ZO-1 phosphorylation	Brain endothelia	[168]
$CdCl_2$	p38	↓ Occludin	Sertoli	[176]
DF	ERK1/2	↓ Occludin, ZO-1 disappears from cell borders	PC-1, PC-1.0, AsPC-1, CAPAN-2	[184,183]
TGFβ3	ERK1/2, p38	↓ Occludin, ZO-1 and Cl-11	Sertoli	[180,176]
EGF+TGFβ1	ERK1/2	↓ Occludin and Cl-1	Thyroid epithelia	[182]
PLGF-1	ERK1/2	ZO-1 disappears from cell borders	ARPE-19	[181]
VEGF	ERK1/2	↓ ZO-1	Peritoneal mesothelia	[178]
TNFα, IFNγ	ERK1/2, p38	↓ Occludin, ZO-1, Cl-2	MDCK	[177]
Mast cell tryptase	ERK1/2	Occludin and ZO-1 disappear from cell borders	Colonocytes	[189]
HIV-Tat protein	ERK1/2	↓ ZO-1 and Cl-5	Brain endothelia	[185,186]
HIV protease inhibitor (ritonavir)	ERK1/2	ND	HMEC	[187]
Matrix metalloproteinases (MMP-7, MMP-9)	ERK1/2	↑ ZO-1 degradation	Pancreatic cancer, brain endothelia	[174,175]
Pseudomona aeruginosa sensing molecule 3O-C(12)-HSL	p38	↓ Occludin and ZO-1	Caco-2	[188]
EPEC	ERK1/2	ZO-1	T-84	[190]

Epithelial cell lines: MDCK, dog kidney; ARPE-19, human retinal pigment; PC-1 and PC-1.0, hamster pancreas; AsPC-1, CAPAN-2, human pancreas. Endothelial cell lines: HMEC, human dermal; HUVEC, human umbilical cord. ↑ Increased or ↓ decreased expression of indicated TJ protein. Cl, claudin.

tectable levels of claudin-2 and an increased expression of claudins-1, -4 and -7 [154]. Moreover, the activation of ERK1/2 by HGF inhibits claudin-2 expression in the leaky strain of MDCK cells (MDCK II) and increases TER, while the treatment of the tight strain of MDCK cells (MDCK I) with an ERK1/2 inhibitor induces expression of claudin-2 and decreases TER by 20-fold [155]. The expression of claudin-2 is particularly relevant since previous studies had demonstrated that the difference in junction leakiness between these two strains of MDCK cells is due to the expression in MDCK II cells of claudin-2, a protein known to form paracellular cationic channels [21,156].

In epithelia it has been observed that treatment with the cytokine IL-17 [157] or with several growth factors including transforming growth factor β (TGFβ) [158,159], epithelial growth factor (EGF) [160] and HGF [155], or the exposure of intestinal cells to bile [161] activates ERK1/2 increasing the barrier function of TJs. Thiols can also promote TJ sealing by activating ERK1/2, JNK and p38. Treatment with glucocorticoids increases TER by recruiting Ras to regions of cell–cell contact and inducing ERK1/2 activation [162]. In endothelial cells endostatin, a 20-kDa fragment of collagen XVIII reverses VEGF-mediated increase in permeability of the blood retinal barrier (BRB), through activation of p38 MAPK and ERK1/ERK2 [163]. Interestingly pretreatment of HUVEC cells with the steroid hormone dihydrotestosterone (DHT), and to a lesser extent with estradiol (E_2), mitigates the permeability defects associated with brief periods of hypergravity [164,165]. In cells subjected to conditions similar to those experienced by astronauts during liftoff, occludin expression is suppressed and permeability increases. Steroid treatment activates ERK1/2 and blocks these effects. However, since pretreatment with DHT induces a longer period of decreased paracellular permeability after hypergravity than E_2, it could explain the higher post-flight orthostatic intolerance (POI) found in female than in male astronauts. POI is the failure of the body to properly adjust to an upright position especially with respect to blood flow, heart rate and blood pressure, and symptoms of POI can develop as a result from fluid leakage through the endothelial cell TJ barrier.

Activation of MAPKs is also involved in TJ opening triggered by Ras [166,154] or Raf transfection [167], treatment with alcohol [168], bile [149,169,170] oxidative stress, [171–173], metalloproteinases [174,175], $CdCl_2$ [176], cytokines TNF∝ and IFNγ [177], growth factors VEGF [178], TGFβ3 [179,180], PLGF-1 [181], and the combined addition of EGF and TGFβ1 [182], the dissociation factor DF [183,184], HIV-1 Tat protein [185,186] and the HIV protease inhibitor ritonavir [187], *Pseudomona aeruginosa* sensing molecule 30-C(12)-

HSL [188], agonist of protease-activated receptor 2 (PAR-2) [189], as well as EHEC [150] and EPEC bacteria [190] (Fig. 5). The deleterious effect of these bacteria on TJ barrier function is inhibited by treatment with the yeast *S. boulardii* that diminishes the bacteria induced activation of the MAP kinase pathway. The deleterious effect of EHEC has also been prevented by pretreating the cells with TGFβ [159].

The action of some of the abovementioned substances is however subject to variation depending on the tissue studied. For example, (1) Ras overexpression triggers in MDCK cells the disappearance of claudin-1, occludin and ZO-2 from cell–cell contact sites due to ERK1/2 activation, while in breast cancer cells no changes in the expression of these proteins are detected [191]; and (2) the addition of bile to rat IEC-6 enterocytic monolayers increases the expression of TJ proteins and diminishes paracellular permeability through ERK1/2-dependent signaling [161], while treating human colorectal adenocarcinoma Caco-2 cells and primary cultures of bovine pancreatic ductal cells with bile acids damages the epithelia and diminishes TER [149,169,170]. This deleterious effect is mediated by generation of reactive oxygen species (ROS) and the subsequent activation of ERK1/2, p38, PI3K and MLCK [149] and can be significantly decreased with EGF [170]. Thus it seems that while the presence of bile in the intestinal lumen favors the normal gut barrier function, its presence in non-physiological sites like the colon or the pancreatic duct is deleterious to the organism. In this respect it should be mentioned that the presence of bile in the pancreatic duct results in an increase in duct permeability for molecules up to the size range of pancreatic enzymes and thereby may contribute to the initiation of gallstone pancreatitis [169,192,193].

A crosstalk between PKC and the MAPK signaling pathway also regulates TJ opening. Thus in corneal epithelial cell lines activation of PKC with PMA triggers a decrease in TER through the activation of MAPK [194].

JAM-1, a type I protein of the TJ characterized for exhibiting two extracellular Ig-like domains, appears to constitute a primary signaling component of the ERK pathway [195]. Thus, JAM-1 silencing hinders the migration of endothelial HUVEC cells on vitronectin induced by basic fibroblast growth factor (bFGF), due to the inability of the growth factor to activate ERK. ERK activation by bFGF is fundamental for triggering

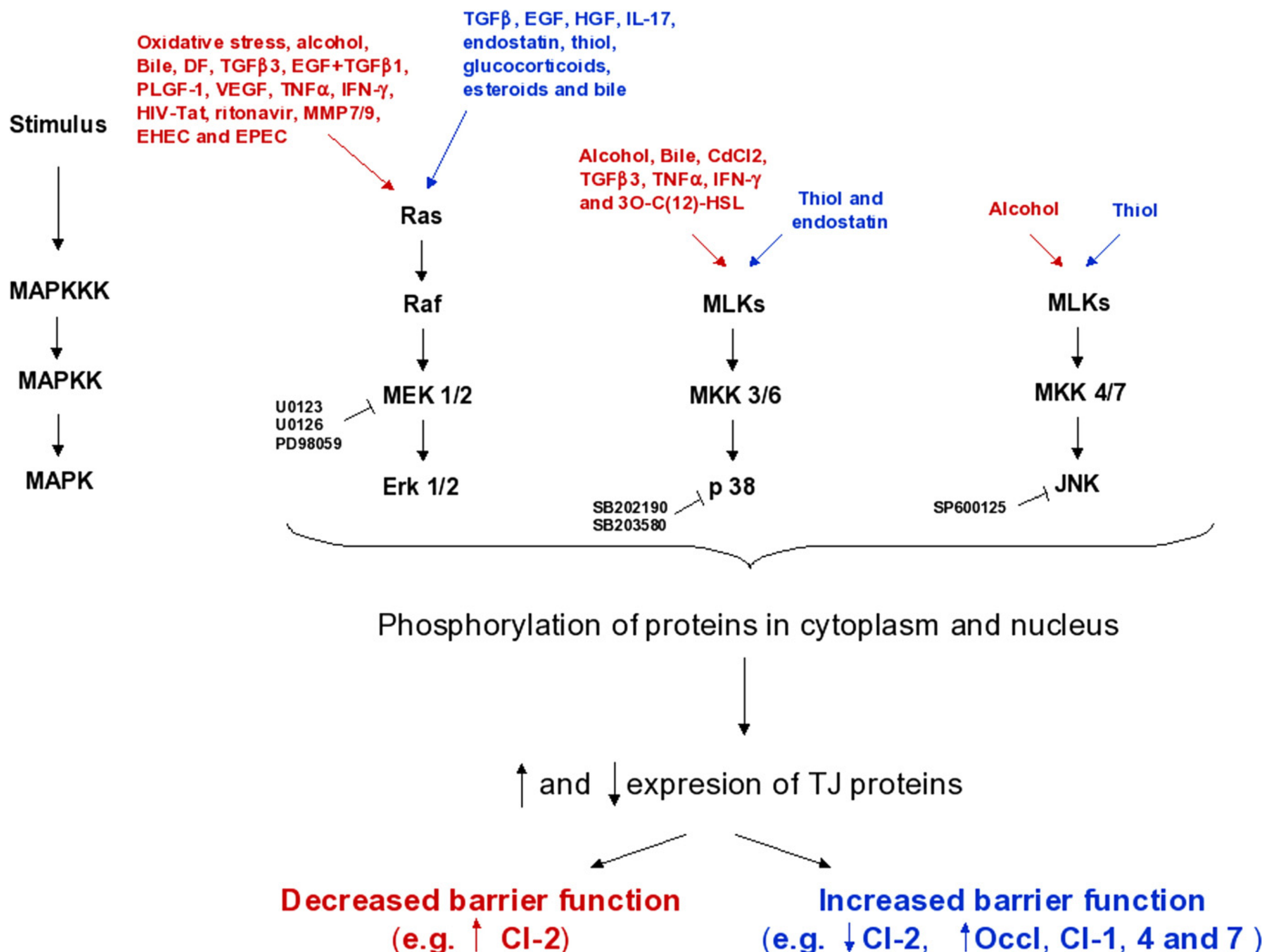

Fig. 5. Activation of the MAPK pathway can lead to TJ opening or assembly depending on the agent that activates the cascade. The uppermost portion of the figure indicates the activators of the MAPK pathway that lead to TJ disassembly (red) or that favor TJ tightening (blue). The hierarchically organization of MAP signaling cascades into three-tiered modules of MAPKKK, MAPKK and MAPK is shown.

endothelial cell migration required for sealing injured vasculature. Interestingly, bFGF signaling involves interaction with integrin $\alpha\nu\beta_3$, which is known to associate with JAM-1 [196].

Occludin also appears to participate in the activation of the MAPK signaling pathway [197]. Thus in hepatic cell lines derived from occludin-deficient mice, MAPK activation is downregulated triggering apoptosis and increased claudin-2 expression. When occludin is transfected into these cultures MAPK becomes activated, reversing the increase in claudin-2 expression and apoptosis.

Therefore we can conclude that the MAPK signaling pathway relates to TJs in two ways: (1) by altering the expression of several TJ proteins and (2) by the interaction of TJ integral proteins with other membrane proteins required for the activation of the ERK signaling pathway.

3.7. PI3K/Akt pathway and TJs

Phosphoinositide 3-kinases (PI3K) are enzymes capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol [198]. Class I PI3Ks are composed of a catalytic and regulatory subunits respectively known as p110 and p85. These kinases convert PI(4,5)P2 to PI (3,4,5)P3 on the inner leaflet of the plasma membrane. The presence of PI(3,4,5)P3 translocates Akt, a Ser/Thr kinase, from the cytosol to the cell membrane. There Akt becomes activated after being phosphorylated at Ser473 and Thr308 by the kinases PDK2 and PDK1. Downstream targets of Akt include the glycogen synthase kinase (GSK-3β), which is responsible for degrading β-catenin, through the ubiquitin proteosome pathway, and snail, a transcription factor that inhibits the transcription of E-cadherin, occludin and claudins. The lipid phosphatase PTEN antagonizes PI3K signaling as it dephosphorylates PI(3,4,5)P3 (Fig. 6). PTEN is a known tumor suppressor, while Akt is involved in cell survival and proliferation.

PI3K associates to ZO-1 and the carboxyl terminal domain of occludin in epithelial cells (Table 1) and its activation affects TJ sealing. For example, PI3K/Akt signaling is involved in the reduced expression of TJ proteins triggered by treatment with HIV-1 Tat protein [186] and TGFβ1 [199,200]. The latter induces an epithelial to mesenchymal transition (EMT) in which the presence of TGFβ1 triggers (1) the Ras/Raf/Mek/Erk pathway that leads to c-Jun activation, AP-1 induced snail transcription, and the subsequent repression of claudins, occludin and E-cadherin expression; (2) the activation of the PI3K/Akt pathway that inhibits GSK-3β blocking β-catenin and snail degradation, generating in consequence the transcription of genes regulated by the LEF-1/β-catenin complex involved in EMT, and repressing the expression of E-cadherin and TJ genes; and (3) binding of TGFβ to its receptor TβRII and its heterodimerization with receptor TβRI, followed by the endocytosis of this complex. In the early endosome TβRI binds to Smad anchor for receptor activation protein (SARA) which recruits Smad2/Smad3. Upon phosphorylation by TβRI the latter are released from the receptor complex and associate to Smad4. This Smad2/3–Smad4 complex then enters the nucleus and promotes the transcription of LEF-1. PI3K is a mediator of Smad signaling since the proper association of Smad2/3 with TβRI requires binding of SARA to PIP3 which is formed by PI3K (Fig. 6).

Occludin expression appears to trigger the PI3K/Akt signaling pathway as activation of this route is downregulated in hepatic cell lines derived from occludin-deficient mice [197].

PI3K signaling is also involved in TJ sealing. Thus in ischemia-injured porcine ileum the PI3K cascade is required for prostaglandin induced recovery of TER and the reappearance of occludin and ZO-1 at intercellular junctions [201], and tightening of TJ barrier induced by glucocorticoids induces the recruitment of PI3K subunit p85 to TJs and is attenuated by PI3K inhibitors [162].

In summary no generalizations can be made on the effect of PI3K signaling pathway on TJ physiology, as activation of this route by different agents can induce opposite effects on the barrier function of TJs.

4. Phosphorylation of specific TJ proteins

4.1. ZO proteins

ZOs are phosphoproteins [43,202]. The analysis of phosphoamino acids from purified ZO-1 [202] or ZO-2 [39] reveals a strong phosphoserine signal. In cells lacking TJs due to the absence of Ca^{2+} in the media, ZO-2 is significantly more phosphorylated in serine residues than in monolayers with well-established TJs. This increased phosphorylation is due to the action of both protein PKC and PKA [39]. Instead a hyperphosphorylation of ZO-2 has been reported to correlate with an increased TER triggered by the PLCγ inhibitor 3-nitrocoumarin [203]. Since this process is reversed by the PKC inhibitor staurosporine, it is assumed to involve Ser/Thr residues. In a similar fashion, the phosphorylation of ZO-1 diminishes when the assembly of TJs triggered by a transfer from LC to NC media is blocked with PKC inhibitors [40].

To our knowledge, the only phosphorylated residue in ZO proteins whose physiological effect has been studied is Ser369 of ZO-2. This residue is located within NES-1 and complies with the consensus for phosphorylation by PKC. ZO-2 contains 4 NES which are Leu-rich sequences containing a characteristic spacing of Leu or other hydrophobic residues. When a peptide corresponding to the sequence of NES-1 was chemically conjugated to the reporter protein ovalbumine and injected into the nucleus, it remained confined to the nucleus, indicating the lack of functionality of the signal [204,205]. However, if Ser369 was substituted by a glutamic acid, the reporter protein left the nucleus through a process sensitive to leptomycin B, an antifungal compound that inhibits the association of NES with the export receptor CRM1/exportin. Furthermore, the substitution of Ser369 for alanine in ZO-2 inhibits the nuclear exportation of the protein [205]. Since the mutation of any of the NES present in ZO-2 is sufficient to block the nuclear exportation of the protein, it is concluded that phosphorylation of Ser369 is critical for the departure of ZO-2 from the nucleus.

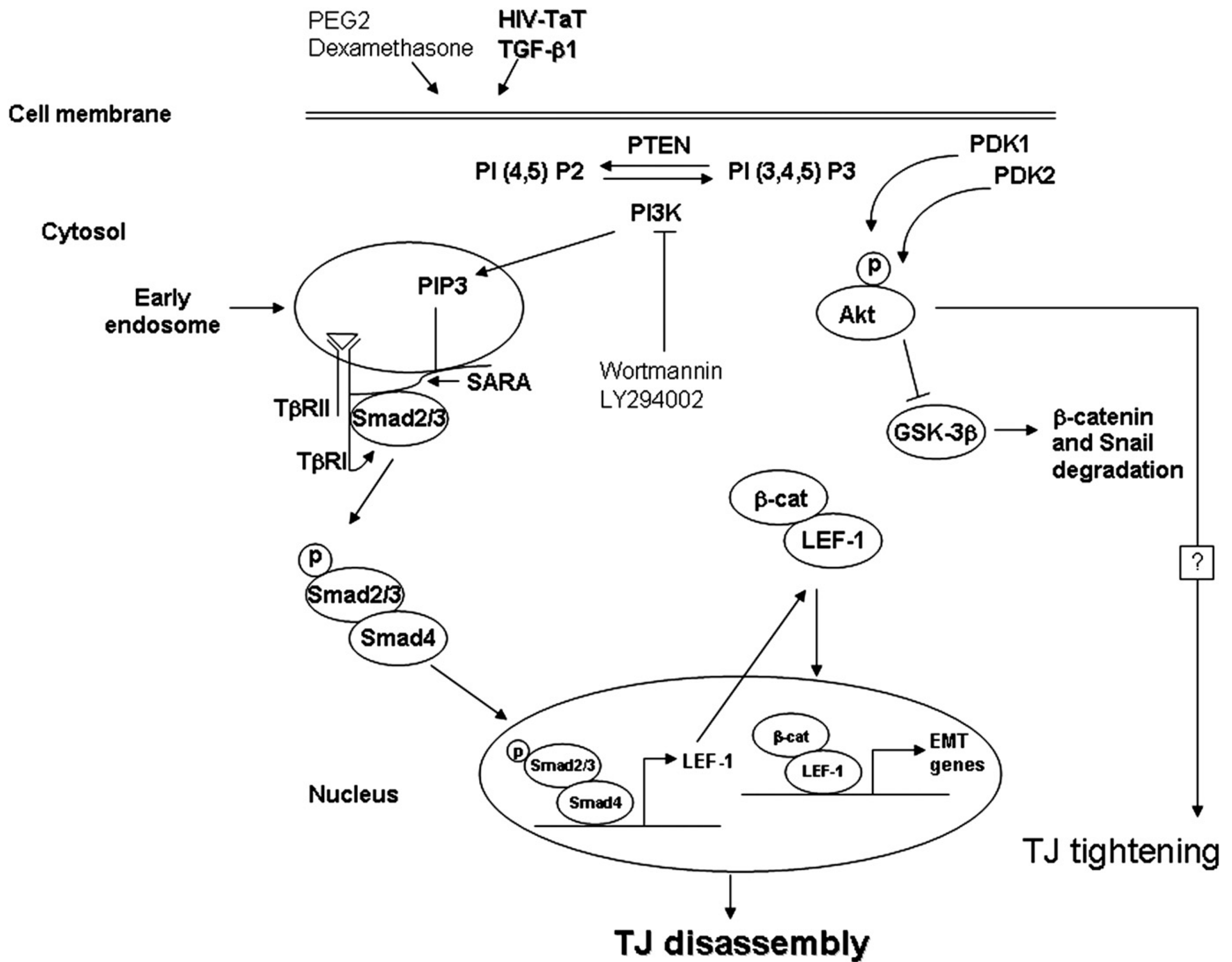

Fig. 6. Schematic representation of the activation of PI3K/Akt kinase pathway and its effect on TJs. The uppermost portion of the figure indicates the activators of the PI3K/Akt pathway that lead to TJ disassembly (bold) or that favor TJ tightening. PI3K generates phospholipids that activate PDK1 and PDK2. These kinases phosphorylate Akt rendering it active. Akt phosphorylation deactivates its target GSK-3β, which as a consequence cannot lead β-catenin and snail to degradation in the proteosome. While snail transcription factor silences the expression of several junctional proteins, β-catenin associates to LEF-1, enters the nucleus and promotes the transcription of genes involved in EMT. The endocytosis of the TGFβ receptor complex requires PI3K as a mediator of Smad signaling that leads to LEF-1 transcription. The signaling cascade downstream of AKT that promotes TJ tightening is not yet known.

ZO proteins can also become tyrosine phosphorylated. Interestingly this phosphorylation occurs both under conditions that favor TJ formation like protamine sulfate treatment of glomeruli [206], EGF treatment in A-431 cells [207], or ATP replenishment in MDCK cells [208], and in others that favor the loosening of the TJ barrier like oxidative stress [209–211], the transfection of v-Src [212], treatment with HGF [213,214] or tyrosine phosphatase inhibitors [215] as well as in colorectal cancer [216].

4.2. Claudins

Claudins are phosphorylation targets of diverse kinases. Next we will describe several cases in which the phosphorylation of claudins alters the barrier function of the TJ, perturbs the morphological asymmetry in embryos or impacts human health.

4.2.1. Phosphorylation of Thr206 in claudin-1 randomizes the direction of heart looping

In vertebrates, the positioning of the internal organs relative to the midline is asymmetric. When a subtractive screen was done to identify genes that are differentially expressed on the left and right side of the chick embryo, claudin-1 was identified as being expressed on the left side. Interestingly, claudin-1 overexpression on the right side of the chick embryos randomized the direction of heart looping. This effect appears to be regulated by claudin-1 phosphorylation, as it is abolished upon mutation of Thr206, a residue that constitutes a putative PKC phosphorylation target [217]. Interestingly this site is conserved as a putative Thr/Ser PKC phosphorylation site in all the species where claudin-1 has been sequenced (Fig. 7A). Furthermore, upon comparing the tail of several claudins we observe that this residue is conserved as a PKC phosphorylation site in claudins-2, -7, -8, -16 and -17 (Fig. 7F).

4.2.2. Thr phosphorylation of claudin-5 by PKA increases the barrier function of brain endothelia and loosens lung endothelial TJs

Claudin-5 although present in certain epithelia [218], is specially concentrated in endothelial cells [27], and in the blood–brain barrier (BBB) plays a crucial role establishing the size discrimination of the TJ [28]. In brain endothelia cAMP increases gene expression of claudin-5, enhances the presence of the protein along cell borders and promotes in it a switch from Ser to Thr phosphorylation via PKA. These effects are accompanied by an elevated barrier function of TJs [106]. Instead, when rat lung endothelial cells are induced to express a claudin-5 with a point mutation in a putative PKA phosphorylation site (Thr207Ala), they become impermeable to inulin (5 kDa) and mannitol (182 Da) while the expression of wild-type claudin-5 allows the passage of the smaller tracer, highlighting how the phosphorylation of Thr207 loosens the size-selective passage of molecules through the TJ of lung endothelial cells.

```
A) Claudin 1 (Thr 203, Thr  206)

Cl-1 Rattus norvegicus   182 LSCSCPRKTTSYPTPRPYPKPTPSTGKDYV 211
Cl-1 Mus musculus        182 LSCSCPRKTTSYPTPRPYPKPTPSSGKDYV 211
Cl-1 Bos taurus          182 LCCSCPRKTTSYPTPRPYPKPAPSSGKDYV 211
Cl-1 Homo sapiens        182 LCCSCPRKTTSYPTPRPYPKPAPSSGKDYV 211
Cl-1 Gallus gallus       182 LCCSCPRSETSYPPSRGYPKNAPSTGKDYV 211
                             *.*****. ****..* *** :**:*****

B) Claudin 5  (Thr 207)

Cl-5 Mus musculus        189 TGRPEFS-FPVKYSAPRRPTANGDYDKKNYV 218
Cl-5 Rattus norvegicus   189 TGRPEFS-FPVKYSAPRRTTANGDYDKKNYV 218
Cl-5 Homo sapiens        189 TGRPDLS-FPVKYSAPRRPTATGDYDKKNYV 218
Cl-5 Gallus gallus       187 LQRDETS-FPVKYSAPRRPTSSGEYDKKNYV 216
Cl-5 Xenopus tropicalis  187 -MKDGISNLPVKYSAPRMPTSNGDYDKKNYV 216
Cl-5 Danio rerio         186 -PASGSSGYSVKYAPTKRATSNGEYDKRNYV 215
                                   *  .***:..: .*:.*:***:***

C) Claudin 16 (Ser 217)

Cl-16 Rattus norvegicus      --------------------------------------------------
Cl-16 Mus musculus           --------------------------------------------------
Cl-16 Homo sapiens       211 DVYVERSTLVLHNIFLGIQYKFGWSCWLGMAGSLGCFLAGAVLTCCLYLF 260
Cl-16 Bos taurus             --------------------------------------------------

Cl-16 Rattus norvegicus  211 --------------------GVSMAKSYKAPRTETAKMYAVDTRV 235
Cl-16 Mus musculus       211 --------------------GVSMAKSYKAPRTETAKMYAVDTRV 235
Cl-16 Homo sapiens       261 KDVGPERNYPYSLRKAYSAAGVSMAKSYSAPRTETAKMYAVDTRV 305
Cl-16 Bos taurus         211 -DVGPERSYPYSTRKAYSTTAVSMPRSHAIPRTQTAKMYAVDTRV 254
                                                 .***.:*:  ***:***********

D) Claudin 3 (Thr 192)

Cl-3 Rattus norvegicus   190 ------YAPTKILYSAPRSTGPGTGTGTAYDRKDYV 219
Cl-3 Mus musculus        190 ------YAPTKILYSAPRSTGPGTGTGTAYDRKDYV 219
Cl-3 Bos taurus          190 ------YARTKIVYSAPRSTGPVTSTGTAYDRKDYV 219
Cl-3 Canis familiaris    189 ----KKYAPTKIVYSAPRSAGPGTST--AYDRKDYV 218
Cl-3 Homo sapiens        191 ------YTATKVVYSAPRSTGPGASLGTGYDRKDYV 220
Cl-3 Xenopus tropicalis  184 CPPKEKYPTSRVAYSAARSTNPG------YDRKDYV 213
Cl-3 Gallus gallus       185 PPKDERYAPSKVAYSAPRSAVTS------YDKRNYV 214
                                   *. ::: ***.**: .       **:::**

E) Claudin 4 (Tyr 208)

Cl-4 Homo sapiens        180 LLCCNCPPR-TDKPYSAKYSAARSAAASNYV 209
Cl-4 Bos taurus          180 LLCFNCPPR-NDKPYSAKYSAARSAPASNYV 209
Cl-4 Rattus norvegicus   181 -LCCNCPPRRNEKPYSAKYSAARSVPASNYV 210
Cl-4 Xenopus tropicalis  180 MLCCNCPPR-DQKPYSAKYTAARSGATSNYV 209
                              ** *****  :*******:****  .:****
```

Fig. 7. Comparison of phosphorylated residues present in claudins sequenced in diverse species. (A) Comparison of Thr203 (rat, red) and Thr206 (chicken, yellow) of claudin-1 with residues present in this segment in other species. (B) Comparison of Thr207 (blue) present in rat claudin-5 with residues present in this segment in other species. (C) Comparison of Ser217 (blue) present in human claudin-16 with residues present in this segment in other species. (D) Comparison of Tyr208 (green) present in human claudin-4 with residues present in this segment in other species. (E) Comparison of Thr192 (blue) present in human claudin-3 with residues present in this segment in other species. (F) Comparison of the phosphorylated residues present in the tail of different claudins from *Homo sapiens*. The highlighted residues correspond to amino acids that constitute putative phosphorylation target sites of the same kinase phosphorylating the boxed residue. The highlighted residue corresponds to the originally described phosphorylated amino acid. Kinase target sites: yellow, PKC; blue, PKA; red, MAPK; and green, EpHA2.

```
F) Homo sapiens claudins tails

Cl-7 Homo sapiens       182 -------------------------------------------LSC---- 184
Cl-1 Homo sapiens       182 -------------------------------------------LCC---- 184
Cl-4 Homo sapiens       180 ------------------------------------------LLCC---- 184
Cl-16 Homo sapiens      211 DVYVERSTLVLHNIFLGIQYKFGWSCWLGMAGSLGCFLAGAVLTCCLYLF 260
Cl-14 Homo sapiens*     210 --------------------------------------------AY---- 211
Cl-14 Homo sapiens      210 --------------------------------------------AY---- 211
Cl-3 Homo sapiens           --------------------------------------------------
Cl-5 Homo sapiens           --------------------------------------------------
Cl-9 Homo sapiens           --------------------------------------------------
Cl-8 Homo sapiens           --------------------------------------------------
Cl-6 Homo sapiens           --------------------------------------------------
Cl-19 Homo sapiens          --------------------------------------------------
Cl-2 Homo sapiens           --------------------------------------------------
Cl-20 Homo sapiens          --------------------------------------------------
Cl-17 Homo sapiens          --------------------------------------------------
Cl-12 Homo sapiens          --------------------------------------------------
Cl-11 Homo sapiens          --------------------------------------------------
Cl-23 Homo sapiens          --------------------------------------------------

Cl-7 Homo sapiens          185 -------SCP-GNESKAGYRAP-----RSYPK--SNSSKEYV----- 211
Cl-1 Homo sapiens          185 -------SCP-RKTT--SYPTP-----RPYPKPAPSSGKDYV----- 211
Cl-4 Homo sapiens          185 -------NCP-PRTDK-PYSAK-------YSAARSAAASNYV----- 209
Cl-16 Homo sapiens         261 KDVGPERNYP-YSLRK-AYSAAGVSMAKSYSAPRTETAKMYAVDTRV 305
Cl-14 Homo sapiens*        212 --------QP-PAAYK-DNRAP----SVTSATHSGYRLNDYV----- 239
Cl-14 Homo sapiens         212 --------QP-PAAYK-DNRAP----SVTSATHSGYRLNDYV----- 239
Cl-3 Homo sapiens          191 -----------YTATKVVYSAPRST-GPGASLGTGYDRKDYV----- 220
Cl-5 Homo sapiens          189 ------TGRP-DLSFPVKYSAPRR---PTAT-G-DYDKKNYV----- 218
Cl-9 Homo sapiens          188 -----PQVER-PRGPRLGYSIP------SRSGASGLDKRDYV----- 217
Cl-8 Homo sapiens          196 -----RYSIPSHRTTQKSYHTG-------KKSPSVYSRSQYV----- 225
Cl-6 Homo sapiens          191 -------QGPSHYMARYSTSAP-----AISRGPSEYPTKNYV----- 220
Cl-19 Homo sapiens         195 ------SPQPYRPGPSAAAREP------VVKLPASAKGPLGV----- 224
Cl-2 Homo sapiens          201 --------QPLATRSSPRPGQP----PKVKSEFNSYSLTGYV----- 230
Cl-20 Homo sapiens         190 ---------P-EARLDPPTQQPISN--TQLENNSTHNLKDYV----- 219
Cl-17 Homo sapiens         195 ------YRYP-VPGYRVPHTDK-----RRNTTMLSKTSTSYV----- 224
Cl-12 Homo sapiens         215 ---------PSMHTYSQPYSAR----SRLSAIEIDIPVVSHTT---- 244
Cl-11 Homo sapiens         178 ------CAGDAQAFGENRFYYT------AGSSSPTHAKSAHV----- 207
Cl-23 Homo sapiens         263 -----VLDGEGWESQDAPSCST-------HPCDSSLPCDSDL----- 292

Phosphorylation targets of different kinases:
MAPK, [red]; PKA, [blue]; PKC, [yellow] and EpHA2, [green]

Residues conservation:
* - single, fully conserved residue
: - conservation of strong groups
. - conservation of weak groups
  -  no consensus
```

Fig. 7 (*continued*).

Although the Thr207 PKA target site present in claudin-5 is conserved in different animal species, it is absent in other human claudins (Fig. 7B and F).

4.2.3. Absence of phosphorylation of claudin-16 at Ser217 by PKA targets the protein to the lysosome

Claudin-16 known as paracellin, concentrates at the TJ of the thick ascending limb of Henle (TAL) and functions as a paracellular pore for Mg^{2+} [22]. This action is crucial for Mg^{2+} homeostasis because Mg^{2+} filtrated in the glomeruli is predominantly reabsorbed by the paracellular pathway in the TAL [219]. Claudin-16 is phosphorylated by PKA at Ser217 under physiological conditions and this phosphorylation is essential for its localization at TJs, as dephosphorylated claudin-16 and the Ser217Ala mutant are translocated into the lysosome [99]. Ser217 is not conserved in claudin-16 derived from other species or in the carboxyl terminal tail of other human claudins (Fig. 7C and F).

A human renal disease known as familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC) is caused by a disfunction of claudin-16 and is characterized by renal wasting of Mg^{2+} and Ca^{2+} [22]. Patients with FHHNC exhibit diverse mutations in claudin-16 that affect the intracellular traffic of this protein. When proteins carrying these human mutations were studied in MDCK cells, they were found to be retained in the endoplasmic reticulum and to undergo proteosomal degradation or to be delivered to lysosomes via clathrin-mediated endocytosis following transport to the cell surface or without appearing in the plasma membrane [220]. These mutations are located in the extracellular loops and the transmembrane domains of claudin-16, therefore they cannot be regulated by phosphorylation. Other cases of lysosomal mistargeting are generated by

missense mutations which delete the entire C terminal cytosolic domain where the critical Ser217 residue target of PKA is located [221]. However, the absence of this residue is not the only trigger for lysosomal mistargeting as mutations that disrupt the PDZ binding motif of claudin-16 are also targeted to the lysosome due to their inability to associate to the TJ scaffolding protein ZO-1 [222,220].

4.2.4. PKA phosphorylation of Thr192 in claudin-3 perturbs the barrier function of TJs

In cancerous tissue, the loss of the epithelial barrier function has been recognized for a long time as an essential step involved in cell transformation [223–227]. Therefore it has been intriguing to find claudin upregulation in several carcinomas (for a review, see [7]), since these proteins are clearly implicated in TJ formation and function. The answer to this conundrum is apparently related to the phosphorylation state of claudins and their *cis*-associated proteins (on the membrane of the same cell). Claudin-3 for example contains a putative PKA phosphorylation site at Thr192 and is an excellent substrate of this kinase. When the ovarian cancer cell line OVCA433 is transfected with a claudin-3 that contains a Thr192Asp mutation that mimics the phosphorylated state, TER does not develop in newly plated monolayers and paracellular permeability increases in mature cultures [97]. These results hence suggest that claudin-3 phosphorylation by PKA may provide a mechanism for the disruption of TJs in ovarian cancer even when claudin expression is upregulated. Thr192 is not conserved in claudin-3 derived from other animal species, or in the carboxyl tail of any of the other human claudins (Fig. 7D and F).

4.2.5. Phosphorylation by MAPK of Thr203 in claudin-1 is crucial for the barrier function of TJs

The putative MAPK kinase phosphorylation site located at Thr203 in claudin-1 is required to promote the barrier function of TJs as a Thr203Ala mutation decreases TER and increases paracellular permeability in rat lung endothelial cells (RLE) [228]. This Thr is conserved in claudin-1 from rat and mouse but not in bovine, chicken and humans and is not found in other human claudins (Fig. 7A and F).

4.2.6. Phosphorylation of claudins-1 and -4 by WNK enzymes generates a chloride shunt in the distal nephron proposed to contribute to the pathogenesis of pseudohypoaldosteronism type II (PHAII)

PHAII is an autosomal dominant disorder characterized by hypertension and high serum potassium levels (hyperkalemia). Reduced renal H^+ secretion is also commonly seen, resulting in metabolic acidosis. The hypertension has been attributed to an increased renal salt absorption and the hyperkalemia to a reduced renal K^+ excretion, despite normal glomerular filtration and aldosterone secretion. Mutations in two homologous Ser/Thr kinase genes named *WNK1* and *WNK4* [With No K(lysine) kinase] are responsible for this disease [229]. Mutations in these genes consist of large deletions in the first intron of WNK1 and missense mutations that cluster within a short span of WNK4. These kinases are expressed in the nephron at the distal convoluted tubule and the collecting duct, segments involved in salt, K^+, and pH homeostasis. WNK1 is cytoplasmic, whereas WNK4 localizes at TJs (Table 1). WNK4 downregulates the activity of the apical thiazide-sensitive Na^+-Cl^- cotransporter and the apical secretory K^+ channel ROMK, the basolateral Na^+-K^+-$2Cl^-$ cotransporter and the Cl^-/HCO_3^- exchanger (for a review, see [230]). When the disease-causing mutants are stably expressed in MDCK cells, they display a gain of function that causes increased paracellular chloride permeability and an enhanced phosphorylation of claudins. WNK4 has been found to increase the phosphorylation of claudins-1 to -4 whereas with WNK1 mutants only the increased phosphorylation of claudin-4 in Thr residues has been explored [231,232]. The presence of such a chloride shunt in the distal nephron is proposed to contribute to the pathogenesis of PHAII. The enhanced phosphorylation of claudins by mutant WNK4 is not due to an increased kinase activity of the mutant, but to an augmented claudin-WNK4 association. The YV PDZ-binding motif found at the carboxyl terminal end most claudins (all except claudins-11, -12, -13 and -16) is necessary for this interaction.

The observation that an increased phosphorylation of claudins exerted by WNK 1 and 4 augments the paracellular permeability of Cl^-, highlights the importance of claudins as therapeutic targets for controlling paracellular charge selectivity. For instance it has been observed that aldosterone which increases the abundance of WNK4 in mammalian kidney, induces in MDCK cells the apical to basal passage of Cl^- and promotes a transient phosphorylation of claudin-4 in Thr residues [233].

4.2.7. Phosphorylation of claudin-4 at Tyr208 by EphA2 enhances paracellular permeability

Another interesting case is posed by the interaction of claudins with the Eph family of receptor protein tyrosine kinases and its ligands. Ephrin receptors and ligands are widely expressed in epithelial cells and their overexpression has been reported in various tumors. Eph receptors are classified in two groups according to their preferential binding to ligands tethered to the cell surface by a GPI anchor (ephrin A) or a transmembrane domain (ephrin B). The interaction of claudin-4, one of the most frequently overexpressed proteins in diverse carcinomas, with EphA2 via their extracellular domains, stimulates the kinase activity of EphA2 and leads to the phosphorylation of claudin-4 at Tyr208. The phosphorylation of this residue contained within the PDZ binding motif of the molecule attenuates the association of claudin-4 with ZO-1, decreasing as a result the integration of this claudin into sites of cell–cell contact and enhancing paracellular permeability [234]. Tyr208 is conserved in claudin-4 from other animal species and in human claudins-1 to -9, -14, -16, -17 and -20 (Fig. 7E and F).

The ligand of Eph receptors B named ephrin B1 is also involved in epithelial adhesion as it interacts in MDCK cells with claudins-1 or -4 on the same surface in cis, via the extracellular domains of these proteins [235]. This interaction that triggers the phosphorylation of ephrin B1 on Tyr residues is markedly enhanced by the trans interaction of claudins upon cell–cell contact formation. Interestingly ephrin B1 phosphorylation that can be abolished by Src kinase inhibitors increases

the degree of paracellular permeability. Therefore these results suggest that the overexpression of claudins in certain cancers would trigger the phosphorylation of ephrin B1, a condition that would in turn generate a leakier TJ.

4.3. Occludin

4.3.1. Expression of different occludin bands depends on the state of cell–cell contact

Occludin resolves by SDS-PAGE, as several bands between 62 and 82 kDa. The lower bands of 62–65 kDa are detergent soluble and phosphorylated in both Ser and Thr residues while the higher ones are insoluble and correspond to forms of phosphorylated occludin [236,237]. The non- or weak phosphorylated forms of occludin distribute along the basolateral membrane while the highly phosphorylated occludin selectively concentrates at the TJ [236].

In the absence of cell–cell contacts MDCK cells only express the lower molecular weight bands. Instead upon TJ formation triggered by switching the cells from low to normal Ca^{2+} media, higher molecular weight bands are expressed [238]. Somewhat similar changes have been observed during the development of mouse embryos, where occludin first assembles at the apicolateral membrane contact site during the 32-cell stage, just prior to blastocoele cavitation [239]. At this time occludin switches form a detergent soluble to an insoluble form indicative of cytoskeletal anchorage. In mouse unfertilized eggs and embryos at different stages of development three major bands of occludin are detected. The lower and higher MW bands of 58 and 72–75 kDa decline in intensity during development, whereas the 65- to 67-kDa band increases in intensity from a minor band in eggs to become the predominant band in blastocysts. Phosphatase treatment has revealed that in blastocysts, the 72–75 and 58 kDa bands are unphosphorylated, while the 65- to 67-kDa band is strongly phosphorylated. In amphibian cells a different situation is encountered, as occludin dephosphorylation correlates with TJ assembly [240]. Thus in *Xenopus laevis* oocytes, occludin is in a phosphorylated form, while fertilization and early embryo development are associated with an electrophoretic downshift of occludin that can be mimicked with phosphatase treatment.

4.3.2. Occludin phosphorylation diminishes during TJ disassembly

In mammalian cells, the overall phosphorylation of occludin is reduced during TJ disassembly, triggered by incubation of the cells in low Ca^{2+} media or by treatment with phorbol esters [237], or by infection with EPEC [241] or *Campylobacter jejuni*, a leading cause of enterocolitis often associated with post-infectious complications including irritable bowel syndrome and Guillain–Barré syndrome [242], and *Burkholderia cenocepacia*, an opportunistic bacteria that in patients with cystic fibrosis traverses the airway epithelium causing bacteremia and sepsis [243].

In cervical epithelium treatment with ATP or diC8, an agonist of conventional and novel PKCs, decreases TJ resistance and generates a breakdown of the 65-kDa occludin to the 50-kDa form. ATP also decreases Thr phosphorylation of the 65-kDa form and increases Thr phosphorylation of the 50-kDa form and Tyr phosphorylation of the 65- and 50-kDa forms [244]. PMA, an activator or conventional and novel PKCs, is remarkable as independently of the cell line used and on its effect on TJs, it promotes Thr dephosphorylation of occludin, suggesting that it targets a Ser/Thr phosphatase which then acts upon occludin [245,76,70].

In Caco-2 cells the phosphorylation of occludin in Thr residues, but not on Ser residues, is reduced during the disassembly of TJs and is gradually increased during reassembly [126], and in LLC-PK1 cells exposure to phorbol esters induces disruption of the TJ associated to a rapid decrease in Thr phosphorylation of occludin [245]. These results contrast with others done in MDCK cells, that show a 71-kDa band of occludin exclusively phosphorylated on Ser residues, that is not present when the TJ is opened by Ca^{2+} removal [237]. The discrepancy could be due to cell-type-dependent differences or to the fact that while in the MDCK studies TJ were disrupted by long-term exposure to low Ca^{2+}; in the Caco-2 cell system a short exposure to the Ca^{2+} chelator EGTA was performed.

4.3.3. VEGF induces the breakdown of the blood–retinal barrier (BRB) and triggers occludin phosphorylation

In retinal endothelial cells (REC), exposure to high glucose results in increased expression of VEGF, as well as the phosphorylation of VEGF receptor-2 (VEGFR-2) [246]. VEGF induces the breakdown of the blood–retinal barrier (BRB) and triggers rapid phosphorylation of occludin and ZO-1 [247]. Accordingly REC of diabetic animals show an elevated occludin phosphorylation [248]. Corticosteroids increase resistance and intraocular pressure [249] and in REC decrease both paracellular flux and occludin phosphorylation [250].

The signaling pathways that underlie VEGF signaling are beginning to be unraveled. Thus, in aortic endothelial cells, VEGF enhances permeability by sequentially involving Src, ERK, JNK and PI-3 kinase/Akt. This leads to the Ser/Thr phosphorylation and redistribution of ZO-1 and occludin. Atrial natriuretic peptide (ANP) inhibits VEGF signaling and TJ protein phosphorylation by interaction with both guanylate cyclase and natriuretic peptide clearance receptors [251].

4.3.4. Rho/ROCK and MLCK signaling regulates occludin phosphorylation

The expression of a dominant negative Rho mutant causes a reduction of the slower migrating, more highly phosphorylated occludin isoforms, whereas a constitutively active Rho mutant causes accumulation of the highly Ser/Thr phosphorylated occludin isoforms [132].

In brain endothelial cells Rho/ROCK signaling causes BBB impairment that involves occludin and claudin-5 phosphorylation in Ser and Tyr residues [252] (Fig. 3). This process appears to be the underlying cause of BBB impairment that allows monocytes migration into the brain during HIV-1 encephalitis. The potent chemokine monocyte chemoattractant protein CCL2 increases BBB permeability, redistributes TJ proteins and generates de novo Ser/Thr phosphorylation of occludin, ZO-1, ZO-2 and claudin-5. cPKCα and aPKCζ participate in this event,

being cPKCα a downstream target for RhoA. Thus brain endothelial permeability can be regulated by monocyte chemoattractant proteins that directly activate aPKCζ or that, through Rho activation, activate cPKCα and ROCK [253] (Fig. 3).

Other substances known to increase endothelial permeability and to promote the phosphorylation of TJ proteins including occludin and claudin-5 are ethanol and its metabolite acetaldehyde [254], as well as oxidative stress stemming from ethanol metabolism [255]. Alcohol induced disruption of the BBB enhances monocytes migration across brain endothelial cells, due to the activation of MLCK that triggers the phosphorylation of MLC and TJ proteins. Lysophosphatidic acid (LPA), a molecule secreted from activated platelets, and histamine trigger the disappearance of perijunctional actin and MLC phosphorylation. Interestingly while LPA activates RhoA and its target ROCK, histamine can increase occludin phosphorylation in a manner independent of both RhoA and ROCK [256].

4.3.5. Occludin is a phosphorylation target of nPKCδ and casein kinases 2 and Iε

In MDCK cells the inhibition of nPKCδ with rottlerin blocks occludin phosphorylation and the formation of the TJ barrier [44]. Thus, treatment of mammalian epithelial cells with the non-phorbol ester agonist of conventional and novel PKCs, bryostatin-1 leads to the appearance of a higher molecular weight form of occludin and increases the barrier function of TJs [81]. Although the precise site of action of nPKCδ on occludin remains unknown, it was previously reported that Ser338 is an in vitro PKC phosphorylation site [257].

Another Ser/Thr kinase involved in occludin phosphorylation is casein kinase 2 (CK2). This kinase phosphorylates two residues that are highly conserved in several species (Thr375 *Xenopus*/403 mouse/405 rat/404 human/403 dog and Ser379 *Xenopus*/407 mouse/409 rat/408 human/407 dog) [258,259]. Casein kinase Iε (CKIε) also phosphorylates occludin and colocalizes and co-immunoprecipitates with occludin from human endothelial cells (Table 1). The regions of occludin sufficient for CKIε binding and phosphorylation are located upstream of the CK2 phosphorylation sites [260]. The impact of occludin phosphorylation by CK2 and CKIε on TJ function is not yet known.

4.3.6. Tyrosine phosphorylation of occludin compromises barrier integrity of TJs

Tyrosine phosphorylation regulates occludin function. For example, treatment of retinal pigment epithelial cells (RPE) with HGF [213] and of brain capillaries and human colonic biopsies with Tyr phosphatase inhibitors [261,262] compromises the barrier integrity of the TJ and increases occludin Tyr phosphorylation. In endothelial cells the hemodynamic force of cyclic circumferential strain decreases permeability and occludin tyrosine phosphorylation and promotes localization of occludin and ZO-1 to the cell borders [263], while shear stress increases hydraulic conductivity and occludin phosphorylation [264].

Moreover, disruption of the BBB by cerebral ischemia [265], or by exposure to glutamate [266], an amino acid that increases dramatically in cerebral ischemia and stroke, augments Tyr phosphorylation of occludin. This process is coincident with an increase in the activity of c-Src [265] and FAK [261] tyrosine kinases.

In endothelial cells oxidative stress and oxidized phospholipids increase paracellular permeability and occludin phosphorylation [267]. In epithelial cells, oxidative stress triggers TJ impairment, tyrosine phosphorylation of occludin and ZO-1, their dissociation from the actin cytoskeleton and redistribution from the junctional area, and reduces their co-immunoprecipitation [211,209]. Oxidative stress induces activation and membrane translocation of c-Src [210]. In vitro tyrosine phosphorylation of the carboxyl terminal tail of occludin by c-Src attenuates the ability of occludin to bind ZO-1, ZO-2 and ZO-3, thus suggesting that c-Src-mediated disruption of TJs involves Tyr phosphorylation of occludin [268]. Oxidative stress also increases the association of occludin with PI3K. The latter is involved in the oxidative stress induced disruption of TJs [209].

In contrast to the abovementioned results that reveal a deleterious effect of Tyr phosphorylation on TJ integrity, during TJ assembly induced in MDCK cells by ATP [208] or Ca^{2+} repletion, occludin becomes Tyr phosphorylated by the action of the non-receptor tyrosine kinase c-Yes [269] (Table 1).

5. Concluding remarks

TJs are dynamic structures regulated by the crosstalk of diverse signaling pathways, whose action varies according to the cellular environment. The availability of stimulators and inhibitors of different steps in the signaling cascades, of dominant active and negative Rho mutants, and of TJ proteins with point mutations at putative phosphorylation sites has ascertained the participation of multiple kinases and phosphatases in finely tuning TJ stability. The identification of TJ proteins as targets of multiple kinases activated under diverse pathological conditions might allow the design of new therapeutic strategies for the treatment of a broad spectrum of human diseases.

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