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Stimulus-induced reorganization of tight junction structure: the role of membrane traffic

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Abstract

The tight junction forms a barrier that limits paracellular movement of water, ions, and macromolecules. The permeability properties of this barrier are regulated in response to both physiological and pathophysiological stimuli, and this regulation has been modeled by pharmacological agents. Although it is now known that vesicular traffic plays important roles in tight junction assembly, the molecular mechanisms by which vesicular traffic contributes to tight junction regulation remain to be defined. This review summarizes recent progress in understanding mechanisms and pathways of tight junction protein internalization and the relevance of these to tight junction regulation.

Keywords

tight junction; tumor necrosis factor; endocytosis; cytoskeleton; myosin; inflammatory bowel disease

Introduction

Complex organisms are composed of multiple organs that serve unique functions. This requires a relatively constant internal milieu as well as the presence of some compartments in which fluid composition is markedly different from the overall milieu. The maintenance of these distinct tissue compartments with defined fluid composition requires barriers to free diffusion. Plasma membranes of individual cells provide some barrier function, but intercellular junctions are needed to seal the paracellular space between cells. Such junctions are present in epithelial and endothelial cells that line surfaces in many tissues within the gastrointestinal, respiratory, and urinary tracts as well as the integument. In each of these tissues, tight junctions join adjacent epithelial or endothelial cells to restrict diffusion, thereby limiting paracellular movement [1]. However, many physiological and pathophysiological situations require exchange of solutes and water between compartments. This demands that the tight junction be able to rapidly regulate barrier function in response to appropriate stimuli [2–4]. While great progress has been made in understanding these processes, the molecular mechanisms by which the tight junction is regulated remains a fundamental question in cell biology, physiology, and medicine.

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Tight junction structure and molecular composition

The tight junction is the most luminal component of the apical junctional complex, which also includes the adherens junction [5]. The latter is composed of cadherin proteins, E-cadherin in epithelia, that form calcium-dependent intercellular bonds [6,7]. Tight junction assembly and maintenance typically requires the presence of intact adherens junctions, though the converse is not true [7–9]. In addition, the structural and functional integrity of the tight junction depends on the presence of a perijunctional ring of actin and myosin which can also contribute to the regulation of paracellular permeability [3,10].

As initially defined by transmission electron microscopy, the tight junction is a site where membranes of adjacent cells appear to nearly fuse [11]. Freeze-fracture electron microscopy shows the tight junction as multiple continuous, anastomosing strands composed of intramembranous particles [12]. Two models have been proposed to explain the chemical nature of the tight junction strands: the lipid model and the protein model. The lipid model proposes that individual tight junction strands represent pairs of inverted cylindrical micelles with the polar head groups of the lipids directed inward and the hydrophobic tails immersed in the lipid matrix of the plasma membranes of both contacting cells [13]. While this model has been generally discarded, the recognition that tight junction membrane domains are enriched in cholesterol and sphingolipids provides some support for the concept that tight junctions are formed, in part, by specialized lipid-based structures. The protein model of tight junction structure (Figure 1) proposes that strands are formed by interactions between integral membrane proteins and peripheral membrane proteins [5,14]. Identification of tight junction integral membrane proteins, particularly the observation that several of these can form strands of intramembranous particles, provides strong support for the protein model [15–18].

Occludin was the first transmembrane tight junction protein to be discovered [19]. Freeze-fracture immunoelectron microscopy showed occludin to be a component of the tight junction strands, and, in fibroblasts, occludin expression induced formation of tight junction-like strands [18,20,21]. Moreover, expression of occludin in fibroblasts induced cell adhesion [20]. Despite this and additional in vitro functional data and in vivo correlative data, the role of occludin in tight junction function has fallen into question because occludin-deficient embryonic stem cells have the capability to develop tight-junction strands indistinguishable from normal strands [22] and occludin-null mice are viable and have normal-appearing intestinal tight junctions [23]. This, however, remains an area in need of further study, as a plethora of in vitro studies do suggest that occludin has important functional roles.

The observation that occludin is not absolutely required for development of polarized epithelial cells bearing normal tight junctions led to a search for additional tight junction proteins. This resulted in identification of the claudin family of proteins [15]. Over 20 claudin family members have been identified in humans where they are localized to tight junction strands and along lateral membranes of epithelial cells [16]. Either individually or in combination with other family members claudin proteins define the charge selective permeability of the tight junction [24]. For example, mutations in claudin-16 prevent paracellular Mg^{2+} reabsorption in the renal tubule, resulting in the Mg^{2+}-wasting disease of familial hypomagnesemia. Interestingly, most claudin-16 mutations result in trafficking defects that limit incorporation of this protein into tight junctions [25]. General trafficking of claudin proteins to the tight junction also requires ZO-1 and ZO-2, which may serve to form a complex linking claudins, occludin, actin, and other tight junction proteins to one another [26,27].

ZO-1 is the prototype of many peripheral membrane proteins localized to the cytoplasmic face of the tight junction [28,29]. Many of these proteins are described as “scaffold” proteins because they interact with both transmembrane and cytoskeletal proteins. Thus, they are

thought to provide some essential part of the regulatory control the perijunctional actomyosin ring exerts on the tight junction, although the details of this are not well understood. In addition to ZO-1, which includes three PDZ domains and an actin binding region, tight junction scaffold proteins include ZO-2, ZO-3, cingulin, and MUPP1 [28,30–33]. A number of kinases, most prominently PKCζ, have also been localized to the tight junction [34–36].

Role of vesicular traffic in tight junction assembly and regulation

Early studies using the calcium switch model of accelerated tight junction assembly showed that apical membrane components accumulated in intracellular vesicles termed the vacuolar apical compartment (VAC) [37]. When extracellular Ca^{2+} concentration was increased to a level sufficient to allow adherens junction assembly, tight junction assembly also occurred and VACs fused with the newly-developed apical membrane. Identification of individual tight junction proteins and preparation of antisera to these led to the conclusion that VACs also contain some tight junction proteins [38,39]. Thus, regulated membrane traffic, which depends on the prior assembly of adherens junctions, appears to be required for tight junction assembly. One exception to this may be the partial polarization of solitary epithelial cells that occurs after activation of STRAD, which activates the serine-threonine kinase mutated in Peutz-Jegher's syndrome, LKB1 [40]. It is notable that STRAD activation only causes incomplete targeting of actin and the cytoplasmic proteins ZO-1 and villin to appropriate locations, while some membrane proteins, such as E-cadherin, are not targeted at al. Data on the effect of STRAD activation on occludin and claudin localization have not been reported.

Endocytosis of tight junction proteins occurs commonly

Given the critical role of membrane traffic in tight junction assembly, it is not surprising that endocytosis of tight junction components also occurs. For over 30 years, electron micrographs have documented the presence of tight junction remnants in cytosolic vesicles, both in cultured cells and in tissues, suggesting that vesicular traffic might be a common mechanism for modification of tight junctions in response to physiological and pathological stimuli [12,41–44]. More recently, occludin internalization has been shown in vitro and in vivo in response to cytokines, bacteria, toxins, and calcium depletion [45–51] (Table 1). We recently visualized the process of occludin internalization after TNF treatment in real time in vivo using mice expressing an EGFP-occludin fusion protein [52]. In addition to these pathological conditions, endocytosis is also seen in physiological conditions and with other type of integral proteins. For example, claudin-3 internalization occurs during movement of epithelial cells within a monolayer [42].

Mechanisms of tight junction protein internalization

The mechanisms that regulate tight junction protein internalization have only been studied in detail over the last decade as transmembrane tight junction proteins were identified and means of detecting individual tight junction proteins have become available. The latter include a wide array of commercial antisera and well-characterized fluorescent fusion constructs of representative tight junction proteins [53]. These tools and growing interest in the role of tight junction disruption in pathophysiology, particularly in the gastrointestinal tract, have spawned a large number of studies evaluating tight junction protein trafficking in response to physiological, pharmacological, and pathophysiological stimuli. Remarkably, all three classic pathways of endocytosis have been reported to be involved in tight junction protein internalization, depending on the stimulus used (Figure 2).

For example, calcium depletion, a non-physiological stimulus that may trigger tight junction disassembly by releasing intercellular cadherin interactions, causes internalization of both adherens and tight junction proteins [50,54]. Immunofluorescent studies suggest that several

proteins, including E-cadherin, β-catenin, occludin, and junctional adhesion molecule-A (JAM-A). These proteins co-localize with one another as well as α-adaptin, a marker of clathrin-mediated endocytosis [50], suggesting that all are internalized via clathrin-coated vesicles. The adherens and tight junction proteins do not appear to remain in clathrin-coated vesicales, as co-localization with markers of early endosomes, such as rab5 and EEA1, is also seen. The colocalization in these early endocytic compartments suggests that the intact complex may be internalized. E-cadherin, β-catenin, occludin, and JAM-A also co-localized with the basolateral marker syntaxin-4, but only E-cadherin and β-catenin co-localized with the Na^+K^+-ATPase. This implies that, once internalized, there is some sorting of junction proteins as well as mixing of basolaterally-derived and junction-derived endosomes, but that this may be differentially-regulated for tight junction and adherens junction proteins [50]. In support of this hypothesis, tight junction and adherens junction proteins appear to segregate from one another in subapical endosomes one hour after calcium depletion. Consistent with the proposed role of clathrin-mediated endocytosis, agents that grossly perturb endocytosis, including acidic and hyperosmotic media, were each able to prevent occludin and E-cadherin endocytosis after calcium depletion. Further support of this interpretation is provided by the failure to detect co-localization of occludin or E-cadherin with caveolin-1 or internalized dextran, or to prevent occludin or E-cadherin internalization with cholesterol depletion or PI3-kinase inhibition, which interfere with caveolar endocytosis and macropinocytosis, respectively. Thus, it appears clear that clathrin-mediated endocytosis of tight junction and adherens junction components occurs after calcium depletion [50]. While it remains to be determined if blockade of clathrin-mediated endocytosis can prevent the loss of tight junction barrier function after calcium depletion, inhibition of myosin II ATPase with blebbistatin, which also prevents occludin and E-cadherin internalization, prevents barrier loss to a limited degree [54]. However, as might be expected of a drug that interferes with actomyosin function, blebbistatin also disrupts the tight junction barrier, even in the presence of extracellular calcium, thereby complicating interpretation of these data.

Interestingly, blebbistatin has also been reported to prevent the occludin internalization induced by interferon-γ (IFN-γ) in cultured intestinal epithelial monolayers [55]. Unlike the endocytosis of tight junction and adherens proteins seen after calcium depletion, IFN-γ induces macropinocytosis-like endocytosis of tight junction proteins [56]. These co-localize with the early endosomes marker EEA1 as well as markers of recycling endosomes, rab4 and rab11, but not lysosomal markers such as LAMP1. It thus appears that, while the general mechanisms of tight junction protein internalization induced by calcium depletion or IFN-γ in cultured intestinal epithelia differ, both involve actomyosin-dependent processes.

Unlike calcium depletion and IFN-γ treatment, the Escherichia coli toxin cytotoxic necrotizing factor-1 (CNF-1), which activates Rho GTPases, seems to induce occludin internalization into caveolin-1-positive structures [49]. This suggests that such endocytosis occurs via caveolae, which are thought to form from lipid rafts and concentrate cargo by nature of the specialized lip[id composition of these domains. CNF-1 also induces redistribution of claudin-1, JAM-A, and ZO-1 though, unlike calcium depletion, these tight junction proteins were not co-localized with occludin and adherens junctions were not disrupted. While internalized occludin did not co-localize with LAMP1, some occludin did co-localize with EEA1 and rab11. The quantitative significance of this was not evaluated, but it does raise the possibility that occludin, and perhaps other tight junction proteins, could be recycled to the tight junction after stimulus-induced internalization. CNF-1 also induced actin reorganization and loss of the pool of phosphorylated myosin II regulatory light chain (MLC) normally present at the tight junction [49,57]. Thus, although intermediates that trigger occludin internalization or barrier loss have not been defined, it appears that the cytoskeleton is also involved in this caveolae-mediated tight junction protein endocytosis. Although the pathway of endocytosis has not been characterized, the noninvasive diarrheal pathogen enteropathogenic E. coli, also causes occludin endocytosis in

vitro and in vivo [46–48]. A large part of this barrier loss is also regulated by cytoskeletal mechanisms, at least in vitro [58,59].

Direct microfilament perturbation causes tight junction protein internalization

Consistent with a central role of actomyosin in regulating barrier function, direct perturbation of filamentous actin with the depolymerizing drug latrunculin A causes tight junction disruption [53,60–63]. Epithelial monolayers expressing fluorescent fusion constructs of occludin, claudin-1, ZO-1, and β-actin and real time fluorescence microscopy with simultaneous measurement of barrier function demonstrated that only occludin internalization correlated with latrunculin A-induced barrier loss [53]. While claudin-1 and ZO-1 were redistributed after latrunculin A treatment, these changes occurred after the bulk of barrier loss, suggesting a secondary event not directly related to barrier loss. Further real time imaging using monolayers expressing red fluorescent-tagged occludin and either green fluorescent-tagged caveolin-1 or yellow fluorescent-tagged clathrin light chain showed that latrunculin A-induced occludin internalization occurred through caveolin-1-positive structures, but not occludin-positive structures. Morphometric and kinetic analyses showed that while occludin trafficked through caveolin-1-positive compartments, relatively little internalized occludin trafficked to EEA1-positive endosomes or LAMP1-positive lysosomes [53]. Moreover, inhibition of endocytosis by cooling to 14°C, hyperosmotic media prevented occludin internalization and barrier loss. Disruption of lipid rafts by cholesterol depletion, which interferes with endocytosis via caveolae, also prevented occludin internalization, but inhibition of Na^+H^+-exchange, PI3-kinase, or clathrin assembly were all without effect [53]. Thus, pharmacological actin depolymerization induces occludin internalization via caveolae and interruption of this process prevents barrier loss. However, these data do not show that occludin is functionally important, only that occludin is a sensitive marker of the endocytic events that accompany actin depolymerization-induced barrier loss. Nonetheless, common themes in tight junction disruption by disparate stimuli appear to include modification of the perijunctional actomyosin ring and occludin internalization by one of several different endocytic pathways.

Relationship of tight junction protein internalization to pathophysiological barrier loss

In recent years, intestinal epithelial barrier loss induced by TNF has received a great deal of attention [35,41,64]. This is, in part, due to the fact that intestinal barrier function is disrupted in Crohn's disease and that TNF neutralization is able to induce disease remission and restore barrier function in patients [65–68]. Thus, it appears that TNF-induced barrier dysfunction may be a critical contributor to human disease [41,67]. Although it had been known for several years that tumor necrosis factor could also induce barrier loss in cultured epithelial monolayers [69,70], the discovery that MLC phosphorylation was markedly increased by TNF represented a major breakthrough in understanding the mechanisms of this process [59]. In addition to a correlation between MLC phosphorylation and barrier loss, specific inhibition of MLC kinase reversed in vitro barrier loss induced by TNF [59]. This TNF-induced barrier loss was associated with enzymatic as well as transcriptional activation of MLC kinase [45,71–73]. Moreover, occludin and claudin-1 internalization were induced by TNF.[45]

Although the mechanisms of in vitro TNF-induced occludin internalization have not been studied in detail, a TNF core family member, LIGHT (lymphotoxin-like inducible protein that competes with glycoprotein D for herpes virus entry on T cells), also induces MLC kinase-dependent occludin and claudin-1 internalization [74]. Detailed morphometric analysis showed that the progressive decreases in tight junction barrier function correlate with increased

numbers of intracellular occludin-containing vesicles [74]. These occludin vesicles also contained caveolin-1, but not clathrin heavy chain. More importantly, inhibition of caveolar endocytosis, but not clathrin-mediated endocytosis or macropinocytosis, prevented occludin internalization and barrier loss [74]. Since inhibition of caevolar endocytosis did not prevent MLC phosphorylation, but MLC kinase inhibition prevented occludin endocytosis and barrier loss, these data also show that MLC phosphorylation precedes and is necessary for occludin internalization and barrier dysfunction, but that caveolar endocytosis is not required for MLC phosphorylation. Thus, like the pharmacological stimulus latrunculin A, caveolar endocytosis seems to be critical to this cytokine-induced barrier dysfunction.

In vivo analysis of stimulus-induced barrier loss and tight junction reorganization

Until recently, analysis of tight junction protein endocytosis in response to defined stimuli had only been studied in vitro. However, recent work has shown that either T cell activation or direct administration of recombinant TNF or LIGHT induces occludin internalization and barrier dysfunction in mice [75,76]. As predicted by some of the in vitro data, this was associated with epithelial MLC phosphorylation and either genetic or pharmacological inhibition of intestinal epithelial MLC kinase prevented both occludin internalization and barrier dysfunction [75]. In an observation only possible in an intact in vivo system, barrier dysfunction induced by T cell activation or TNF administration resulted in net intestinal water secretion, i.e. diarrhea, which was also prevented by either genetic or pharmacological MLC kinase inhibition [75,76]. In contrast to TNF, LIGHT, which induced similar MLC kinase-dependent barrier dysfunction, actually enhanced water absorption [76]. This difference was due to the second effect of TNF, downregulation of epithelial Na^+ absorption, which was not induced by LIGHT. Thus, in addition to demonstrating that MLC kinase-dependent barrier dysfunction is necessary for cytokine-driven acute diarrhea to occur in vivo, these data also demonstrate that the barrier dysfunction is only part of the story. Water can flow in either direction through tight junctions in which cytokines have caused increased permeability. The determination of the direction of water flow depends on osmotic gradients generated by active transepithelial transport [76,77].

The in vivo studies also show that, despite MLC kinase-dependent occludin internalization (Figure 3), endocytosis of ZO-1, claudins, and adherens junction proteins did not occur [75]. Occludin endocytosis without claudin endocytosis is similar to that induced by latrunculin A in vitro, but contrasts sharply with the other in vitro studies which generally showed internalization of occludin along with claudin, JAM-A, or other tight junction proteins. While it remains to be characterized, these disparate results may well reflect the difference between acute or transient stimuli and chronic stimuli. In the case of latrunculin A, claudin-1 redistribution was seen at later time points, after most barrier function losses had occurred. Thus, if only the endpoint, rather than many intermediate time points, were assessed, the results might have been mistakenly interpreted to imply that claudin redistribution coincides with barrier loss. In the in vivo example given a pulse of cytokine release occurs and then rapidly diminishes. Thus, it may be that prolonged cytokine simulation, as occurs in chronic disease, may induce internalization of claudin proteins as well as occludin. This, in fact, has recently been observed in epithelia from patients with Crohn's disease [78–80].

The temporal correlation of occludin internalization with initial barrier loss also suggests that occludin may be a critically important functional component of the tight junction. However, reports that occludin knockout mice have normal intestinal barrier function have been interpreted to imply that occludin does not play an important role in tight junction structure or regulation [23,81]. While no in vivo data are available to refute this interpretation, the fact remains that abundant in vitro data clearly support a functional regulatory role for occludin.

These include data from epithelial cells in which occludin expression was suppressed by stably expressing short interfering RNA [82]. Thus, it may be that the few studies of intestinal function in occludin knockout mice have failed to show a functional deficit because the mice have not been subjected to stress or, alternatively, because there are compensatory changes in expression of another, as yet unidentified, protein. Clearly, this is an area in which more work is needed.

Concluding comments

It is now clear that vesicular traffic plays an important role in regulating tight junction structure and function in response to a wide variety of stimuli. The demonstration that this process also occurs in vivo, and is specifically-related to intestinal disease, makes it essential that these observations be defined in greater detail. For example, although various pathways of endocytosis have been described morphologically, few studies have shown that these events are absolutely required for regulation of barrier function. The molecular events that trigger endocytosis and how these relate to tight junction structure are also in need of further characterization. Nonetheless, the available data argue strongly that the tight junction is a far more dynamic and complex structure than previously recognized. Enhanced understanding of these processes may provide means to selectively and specifically regulate the tight junction and allow development of targeted therapies for diverse diseases of barrier function.

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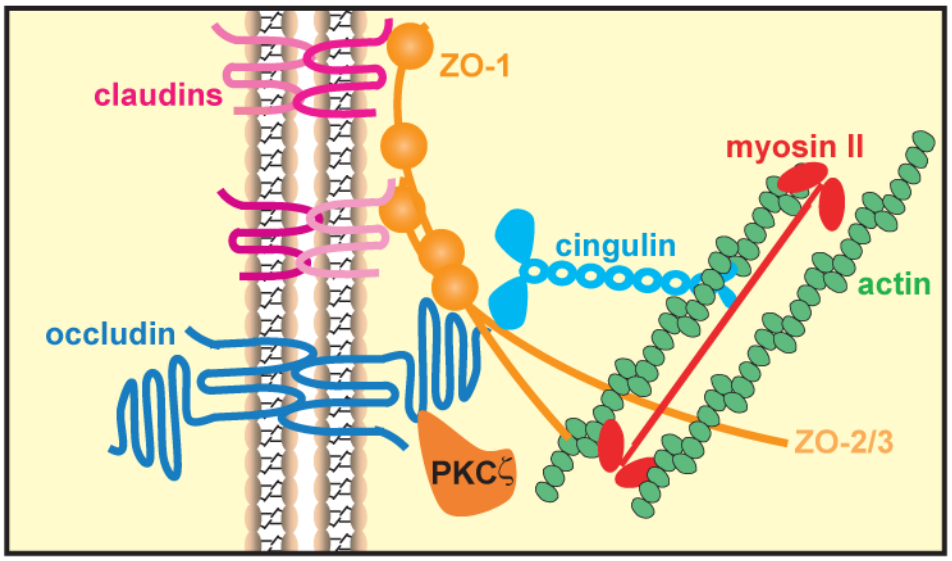

Figure 1.
Molecular composition of the tight junction. Tight junctions consist of three main groups of proteins. They are transmembrane proteins, e.g. claudins and occludin, cytoplasmic plaque proteins, e.g. ZO-1, ZO-2, ZO-3, and cingulin, and cytoskeletal and signaling proteins, e.g. actin, myosin II, and PKCζ. These proteins are interact to maintain tight junction structure.

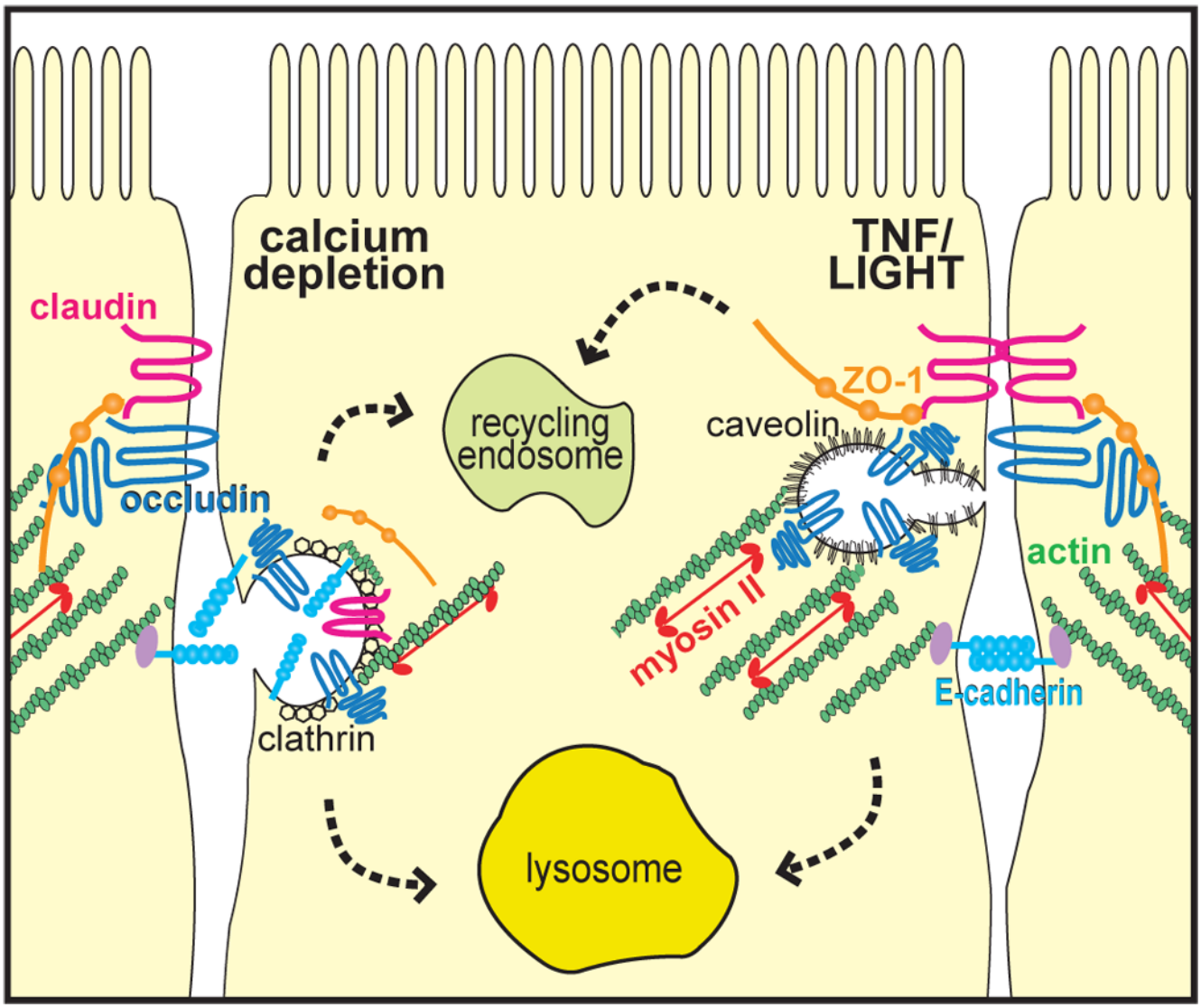

Figure 2.
Stimulus-induced endocytosis of tight junction proteins. Calcium depletion causes clathrin-mediated endocytosis of adherens junction (E-cadherin) and tight junction (claudin, occludin) proteins. TNF core family members caused caveolae-mediated endocytosis of occludin, but not other proteins. Both pathways involve actomyosin-dependent processes. The fate of these vesicles, including whether they fuse with lysosomes, resulting in protein degradation, or with recycling endosomes, with protein trafficking back to the tight junction, remains to be defined.

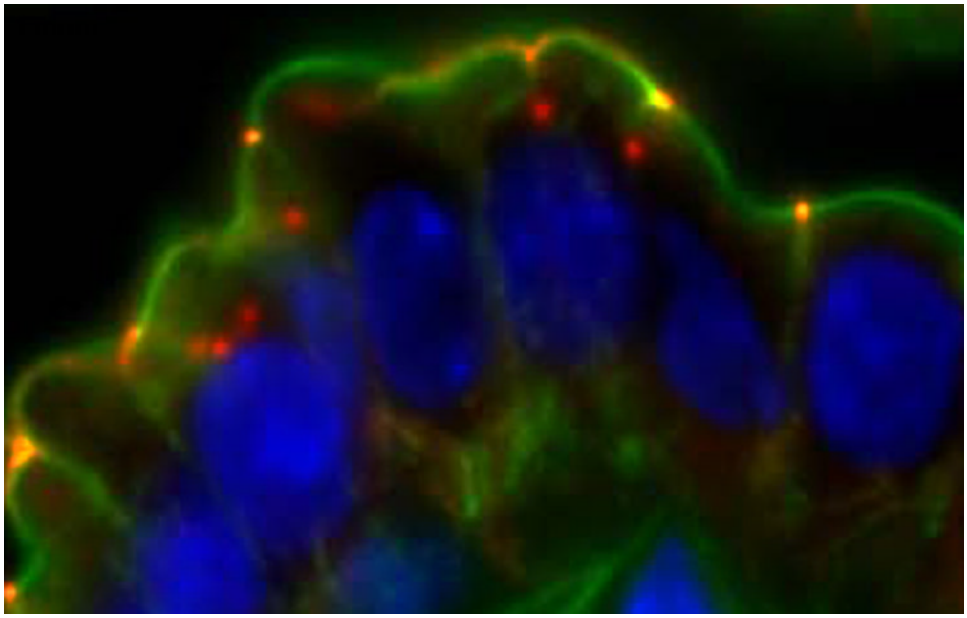

Figure 3.
Occludin is internalized in vivo after immune activation. T cells were activated in vivo by intraperitoneal injection of anti-CD3 antibody. This resulted in TNF-dependent occludin (red) internalization. F-actin (green) and nuclei (blue) are shown for reference.

Table 1

Mechanisms of stimulus-induced regulation of tight junction structure.

stimulus	cell line/tissue	endocytic pathway	signaling event	cytoskeletal event	vesicle content	reference
E. coli CNF-1 toxin	T84	caveolae		rhoA, rac1, and cdc42 activation, MLC dephosphorylation	occludin, claudin-1, JAM-A, ZO-1	[49]
Enteropathogenic E. coli infection	T84		ezrin phosphorylation	MLC phosphorylation	occludin, claudin-1	[47,48,58, 83]
Enteropathogenic E. coli infection	mouse jejunum and colon				occludin	[46]
Clostridium difficile toxins	T84	caveolae	PKC a/β activation	rho GTPase inactivation	occludin, ZO-1	[84–86]
Calcium depletion	T84	clathrin	adherens junction disruption	myosin ATPase activation	occludin, JAM-A, claudin-1, ZO-1	[50,54]
IFN-γ	T84	macropinocytosis	rho kinase	myosin ATPase activation	occludin, JAM-A, claudin-1	[55]
Helicobacter pylori CagA	MDCK		c-Src inactivation	cortactin dephosphorylation	ZO-1, JAM	[87] [88]
latrunculin A	MDCK	caveolae		actin depolymerization	occludin	[53]
TNF	Caco-2		MLCK activation	MLC phosphorylation	occludin, claudin-1	[45]
LIGHT	Caco-2	caveolae	MLCK activation	MLC phosphorylation	occludin, claudin-1	[74]
T cell activation, TNF, or LIGHT	mouse jejunum		MLCK activation	MLC phosphorylation	occludin, JAM-A	[75,76]