Molecular Physiology and Pathophysiology of Tight Junctions III. Tight junction regulation by intracellular messengers: differences in response within and between epithelia

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The aim of the present article is to draw attention to some findings related to the regulation of the permeability of the tight junctions. The message we want to stress is that we should define changes in tight junction permeability with respect to the permeating substances. The often-used electrical resistance is just one characteristic of permeability; it may increase without effect on permeability of large, possibly immunoreactive or toxic, molecules, whereas a severalfold increase in permeability for proteins may occur without noticeable change in electrical resistance. The answer to these discrepancies could lie in the postulated nature of the tight junctional permeability, which may be considered as a large array of parallel conductances consisting of size- and charge-limiting pores within the tight junctional strands. An increase of the number of small pores would decrease the resistance without an increase in permeability for large molecules that cannot pass the pores. If there are just a very few large pores among a huge number of small pores, a doubling of the number of large pores would double the flux of large molecules but would not change the permeability for molecules that can pass through the small pores measurably. Alternatively, the possibility that a few small pores fuse to a larger pore can be considered.

We focus the discussion on effects that are considered to depend on activation of protein kinase A (PKA) and protein kinase C (PKC) in confluent monolayers with established tight junctions or native intestinal preparations. Thus far, it appears that in intestinal epithelia activation of PKA may increase the ionic conductance of tight junctions without changing the barrier function for large molecules. Activation of PKC, however, may increase the permeability for large molecules. In particular, because a large number of bacterial toxins and possibly viral proteins appear to activate the PKC pathway (see references in Ref. 14), this mechanism may be of great importance in generating disorders of the intestinal barrier.

SINCE THE DISCOVERY of the first of the tight junction-associated proteins (ZO-1) a large number of cytoplasmic peripheral proteins have been described that form a complex in a tight junctional plaque. More recently, transmembrane proteins have been added to this complex. Their importance in tight junction function as gates between luminal and blood sides in epithelia has been illustrated by, for example, expression of mutants. Immunoprecipitation techniques allow the study of the association of the different constituents. Earlier studies have dealt with the molecular architecture of the junctions. However, despite the large leaps forward in our knowledge of the constituting elements we are still floundering about the regulation of the gates.

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ION SELECTIVITY OF TIGHT JUNCTIONS AND MODULATION BY PKA

Because the intestinal epithelial cells of fish do not express cAMP-modulated Cl channels like cystic fibrosis transmembrane conductance regulator (CFTR), that tissue was the first in which it could be shown that cAMP could modify the ion selectivity of the tight junctions. Like those in many other epithelia, the tight junctions are cation selective in fish. An increase of intracellular cAMP reduces the cation selectivity and reduces the transepithelial resistance without a change in the cell potential.

One method to illustrate this effect of cAMP on tight junctions is to pass relatively large electrical currents through epithelium mounted between two NaCl-containing solutions. At least 95% of this current is driven through the paracellular pathway. At the serosal border of the lateral intercellular space, Cl carries most of this current because of its larger mobility in water, whereas at the tight junctional border Na carries most of the current because of the cation selectivity of this structure. This so-called transport number effect leads to accumulation of NaCl in the lateral intercellular space or to depletion of the salt, depending on the direction of the applied current. This leads to a decrease or an increase of the transepithelial resistance, respectively. The difference in transport number in the tight junction border and the basement membrane border becomes smaller when the ion selectivity of the tight junctions is decreased, that is, when the conductance for Cl is increased. This leads to an attenuation of the salt accumulation or salt depletion when current is passed.

Experimentally, no change in resistance could be generated when cAMP was already high in the cells before the current was passed. When the resistance has been increased by serosa-to-mucosa current under control conditions, the application of cAMP-generating compounds to the epithelium reduces the resistance. With this experimental approach it has also been shown that cAMP can affect the Cl conductance of the tight junctions in rat ileum (6). Similarly, but with other methods, it has been shown that the Cl permeability of tight junctions, and thereby the transepithelial conductance, is increased in cultured human colonic cells: by vasoactive intestinal polypeptide (VIP) in T84 cells (9) and by forskolin in HT29cl.19A cells (1) and in a Caco-2 clone that does not express Cl secretion when forskolin is applied (J. Karczewski and J. Verheul, unpublished observations). In the intestine of seawater fish cGMP appeared to be more effective than cAMP (30).

Not all cAMP-mediated effects on the paracellular pathway are caused by changes in the tight junctions. For instance, in Necturus gallbladder cAMP does not modulate the tight junction conductance but changes the resistance of the lateral intercellular space (17). An intriguing observation is that the anion selectivity of the increase of the tight junction conductance is like that of CFTR and that the permeability for mannitol is hardly affected. The regulation is rapid (within minutes) and reversible. The Rp diastereomer of adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS) inhibits the effect of VIP, indicating the involvement of PKA and possibly the phosphorylation of an unknown target. A reasonable guess would be that PKA-mediated cytoskeletal changes could affect the dimensions of the tight junctions via myosin-actin interactions with the peripheral tight junction protein complex comprising ZO-1, -2, and -3 and other proteins that are connected to the transmembrane tight junction proteins occludin, claudin, and junctional adhesion molecule (JAM) and probably other integral proteins. It has been shown that the phosphorylation of myosin light chain (MLC) and the subsequent contraction of basal actin-myosin bundles are crucial to the cAMP-driven activation of the Na-K-2Cl cotransporter and the subsequent apical Cl efflux (15). The two cAMP-mediated effects, namely increase of Cl secretion and increase of Cl conductance in the tight junctions, may thus be parallel effects of activation of the cytoskeleton. Cytochalasins, which affect the actin skeleton, have been shown to modify the tight junctional structure and function. However, a comparison between the effects of cytochalasin D and cAMP showed that the cAMP effect was much faster, readily reversible, and much more specific (4).

Phorbol esters and Ca (see below) may also affect the cytoskeleton via PKC. However, in HT29cl.19A cells, time analyses of the changes in resistance on forskolin application and on Ca ionophore or phorbol ester application showed that the rapid decrease of the paracellular resistance occurred only with forskolin (1, 2). Thus the fast effect on Cl conductance of the tight junctions may be specific for an effect of PKA and suggests that one or more tight junction-related proteins may be direct substrates for PKA. The resulting change may be such that existing water-filled pores, lined with negative surface charges, increase their diameter just a little, so that permeability for other molecules is hardly affected, or decrease their surface charges. Both lead to facilitation of Cl permeation. Alternatively, Cl-specific ion channel-like structures not already open may be activated by action of PKA. To our knowledge, PKA effects on the transmembrane tight junction proteins occludin and claudin have not been described. Although occludin can be phosphorylated, the kinase(s) involved are not yet disclosed, and it may well be that occludin and claudin are not direct substrates for PKA (8). The exciting discovery of paracellin-1 (26), a member of the claudin family, in the thick ascending limb of Henle lends strong support to the idea that the tight junction strands may contain ion channel-forming proteins. Paracellin-1 is a divalent cation-specific tight junction protein with a strong negative charge. The permeability of the thick ascending limb for Mg and Ca, and thereby the reabsorption, is physiologically regulated. The discovery of this specific protein may open the way to study the cellular regulation of tight junction permeability for Mg and Ca and, hopefully, of general mechanisms.
involved in the process of regulation of the conductance of tight junctions.

Although in the epithelia mentioned above no effect on monovalent cation conductance by cAMP was observed, there is at least one example in which cAMP affects Na conductance. For instance, low-resistance Madin-Darby canine kidney (MDCK) cells grown on coverslips show increased Na permeability of their tight junctions after application of the PKA-activating Sp diastereomer of adenosine 3′,5′-cyclic monophosphothioate (Sp-cAMPS), whereas the conductance was decreased on application of the PKA-inhibiting Rp-cAMPS (19). These observations were made on individual tight junctions by measuring the rate of disappearance of Na from the lateral intercellular space into the low-Na apical compartment using the fluorescence of sodium-binding benzofuranoxazole (SBFO) as a Na indicator. An important observation in these cells was that the PKA-dependent regulation of the tight junction permeability was in parallel with other processes regulating the actin cytoskeleton. Thus these authors suggest that the cytoskeleton is not involved in the PKA-mediated change in ion permeability of the tight junctions.

One of the strengths in the technical approach of Kovbasnjuk et al. (19) is that they can observe effects on one individual tight junction. This differs from the measurements of fluxes or dilution potentials or the effect of current injections, in which the results show the mean of effects on all tight junctions and sometimes also the cell membranes. Therefore, because of the lack of detail, one can only consider the tight junctional permeability as though it reflects a number of parallel and uniform pores.

TYROSINE KINASE AND PHOSPHATASE

Experiments using inhibitors of phosphotyrosine phosphatase or tyrosine kinase have shown that increased tyrosine phosphorylation increases the tight junction permeability in MDCK cells. Although occludin may be heavily phosphorylated at its tyrosine sites, there is only a very limited number of reports (see Ref. 24) relating protein tyrosine phosphorylation to permeability in intestinal epithelia. The reason may be that on full maturation of the cells, adherens junctions are uncoupled from tight junctions and thereby permeability becomes independent from tyrosine phosphorylation (13).

PROTEIN KINASE C

The modulation of the permeability of tight junctions by phorbol-activated PKC has been documented in a number of cell lines. In MDCK cells 12-O-tetradecanoylphorbol-13-acetate (TPA) induced a large increase in permeability of tight junctions accompanied by alterations in cell morphology; the latter could be prevented by cytoskeletal active drugs such as cytochalasin D. The effect on permeability, however, was not sensitive to these drugs (22). Thus the cytoskeleton is not directly involved in phorbol ester-induced permeability of tight junctions (25). TPA reduced the phosphorylation level of occludin at serine and threonine and concomitantly changed the localization as a continuous band in the cell circumference into a discontinuous pattern along the cell periphery (11).

Extensive work has been done in another kidney cell line, LLC-PK1, since 1986 (21), in which it has been shown that a relatively short incubation period with TPA induced a large increase in permeability for molecules as large as ruthenium red (mol wt 786). The PKC inhibitor GF-109203X can prevent this. The expression of a dominant-negative PKC-α made the cells insensitive to TPA, suggesting that PKC-α may be involved, and, more recently, this group showed that overexpression of PKC-δ made the monolayer more permeable, as after TPA activation (20).

In T84 cells, which hardly express PKC-α (3), application of the Ca ionophore A-23187 increased the paracellular permeability for mannitol and decreased the transepithelial resistance. The effect was inhibited by protein kinase inhibitors such as H7 and sphingosine but not by W13, implying that calmodulin kinase was not involved. Application of phorbol ester phorbol 12-myristate 13-acetate (PMA) mimicked the effect of A-23187, but only after a time delay in which the resistance was increased (29).

An increase of resistance has also been observed in the LLC-PK1A clone (10) and in Na-glucose cotransporter 1 (SGLT-1)-transfected Caco-2 cells (31). In this clone, phorbol ester strongly reduced MLC phosphorylation but increased the phosphorylation of MLC kinase (MLCK). Thus phosphorylation of MLCK may inactivate the enzyme and thereby decrease the phosphorylated state of MLC, which may reduce the tension of the perijunctional actomyosin ring and decrease the width of pores in the tight junction.

In contrast, another report dealing with Caco-2 cells shows that phorbol ester induced a decrease of resistance and an increase of mannitol flux. Carbachol in the presence of a diacylglycerol kinase inhibitor, monolein, mimicked the phorbol ester effect (28). The difference may be caused by differences in development of the preparations or in the clones used. Now that tools have become available to probe the function of the members of the PKC family, it is expected that differences in performance of clones and individual tight junctions can be analyzed in relation to the specific PKC isotypes.

Little is known about the effects of phorbol ester on permeability in isolated intestine. In native intestinal preparations the study of effects of phorbol ester is complicated by effects of the anesthetic pentobarbital (27). However, a barrier-decreasing effect of phorbol esters in rat colon (27) and rat jejunum (23) has been described.

In isolated rat ileum, carbachol does not change the electrical resistance but increases the permeability for the macromolecular probe horseradish peroxidase (HRP) severalfold, whereas the permeability for mannitol is increased by only 75%. Comparison of the flux
of three probes with different molecular weights and electron micrographs showed that the increase of the permeability was in the paracellular pathway. HRP could be detected in some but not all tight junctions. This effect was not found with forskolin, although forskolin reduced the electrical resistance. Thus changes in electrical resistance or mannitol flux do not predict the magnitude of permeability increase for macromolecules (7).

Furthermore, it has been shown that stress can increase the macromolecular permeability of the intestine, which could be prevented by atropine injection before the stress stimulus. This suggests that stress may induce an increased release of ACh in the intestinal wall and that the activation of muscarinic receptors in the tissue leads to increased permeability directly via the enterocytes and/or indirectly via mast cells (5, 16).

The acetylcholinesterase (AChE) inhibitor pyridostigmine, used as a pretreatment under the threat of chemical warfare and in myasthenia gravis, has been related to the decreased function of the blood-brain barrier during stress (12). One may think of a more pronounced rise in ACh levels and therefore a stronger effect on muscarinic receptors. Carbachol does not increase the permeability for macromolecules in HT29cl.19A cells, although in these cells carbachol activates PKC-α (3). However, phorbol activation of

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**Fig. 1.** The effect of phorbol-12,13-dibutyrate (PDB) on the local permeability of Dextran 4400. Caco-2BBAm cells were cultured for 3 wk on Falcon filters. The monolayers were treated over 4 h with 1 μM PDB. During the last 15 min, Texas red-conjugated lysine-fixable dextran (molecular size 4.4 kDa; TR-3, Molecular Probes) was applied to the apical bath. Next, cells were fixed with 4% paraformaldehyde and permeabilized using PBS containing 0.2% Triton X-100, 1% normal goat serum, and 0.1% sodium azide. ZO-1 was stained at 4°C overnight using a polyclonal antibody (Zymed 61-7300). ZO-1 was visualized by fluorescent antibody staining using Alexa 488-labeled secondary antibody (Molecular Probes). The filters were embedded in Vectashield (Vector Laboratories) to minimize the bleaching of dyes. Confocal images were obtained using a Noran OZ system (Noran Instruments) consisting of a Zeiss Axioskop with Plan Neofluar ×40 NA 1.3 oil objectives and a Silicon Graphics O₂-based analysis platform with Intervision 2D and 3D reconstruction software. A: an x-y cross-section at the level of the tight junctions. ZO-1 is stained in green and dextran in red. The localization of ZO-1 was locally disturbed by PDB. In the middle of the image, ZO-1 staining is interrupted and blurry. The outer part of the image shows a regular pattern of ZO-1 that is similar to control monolayers. The horizontal red line in A indicates the position of the x-z cross-section displayed in B. Positions I and II are indicated in the cross-section by arrowheads. Position I shows that dextran can pass the apical barrier and reach the lateral space, which is not seen in control monolayers. Position II is at a visually normal ZO-1 staining, and here there is no passage of dextran. C: pixel intensities at positions I and II along the z-axis.
PKC induced an increased permeability for HRP (18). One obvious possibility is that phorbol ester activation covers a broader range of PKC isotypes than carbachol. These questions can be solved in the future as probes become available to inhibit specific isotype functionality.

With probes and imaging techniques to detect displacements of PKC isotypes and tight junction proteins together with permeability markers, the reason for inhomogeneity in response of tight junctions can also be studied. For instance, in Fig. 1 the increase of the permeability in some but not all tight junctions by phorbol ester in Caco-2BBe cells is shown. This approach can be combined with overall flux measurements and electrophysiology and allows a more detailed analysis of the control mechanisms of paracellular permeability. It may also solve questions about high permeability in disease states. Is the high permeability caused by increased activity of the intracellular messenger system (e.g., activation of PKC isotypes), or is it caused by loss or overexpression of structural tight junction elements? The pathophysiology may help to increase understanding of permeability regulation and defects (cf. paracellin-1). This will require technical and logistical cooperation between science-minded clinical physicians and experimental scientists.

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REFERENCES


