Structure of tight junctions in epithelia with different permeability
(freeze-fracture/electron microscopy/epithelial transport/transepithelial resistance)

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ABSTRACT Freeze-fracture studies have shown a network of intramembrane fibrils in the tight junctions of epithelia. A direct correlation between the number of fibrils and junctional permeability has been suggested by previous studies. However, we have made two groups of observations showing that junctional permeability is not univocally related to the complexity of the network revealed by freeze-fracture. (i) The tight junctions of the rabbit ileum mucosa are permeable to lanthanum, although they have a complex network of fibrils resembling the junctions of toad urinary bladder, which are impermeable to lanthanum. (ii) The tight junctions of the toad urinary bladder are normally of low permeability; however, when the luminal solution is made hypertonic with lysine, junctional permeability markedly increases and lanthanum permeates through the tight junctions. In freeze-fracture replicas, no differences between the fibrils of control and lysine-treated bladders were found. Our results indicate that junctional permeability is controlled not only by the complexity of the fibrilar network, but that some features of the junctions or the fibrils themselves, not yet revealed by electron microscopy, play a central role in regulating epithelial permeability.

Tight junctions are specialized cellular contacts that seal lateral intercellular spaces at the apical side of lining epithelia. Electron microscopy has shown that the close apposition between adjacent plasma membranes at the tight junction, also termed zonula occludens, results in the obliteration of the intercellular cleft, forming a continuous sealing belt around epithelial cells (1).

Freeze-fracture studies, which expose frozen membranes along the central plane of the bilayer (2), show that in the region of the tight junction there is a branching network of intramembrane “fibrils” (3-6). These junctional fibrils span the thickness of the adjacent membranes (4, 7). It is of interest to elucidate to what extent the focal obliteration of the extracellular space depends on the organization of the junctional fibrils. There are two obvious approaches to the tackling of this problem. On the one hand, it has been established that the permeability of tight junctions as determined by physiological criteria, such as transepithelial resistance and permeability to large hydrophobic solutes, and by morphological studies, varies considerably among different epithelia (8-12). A correlation between normal variations in tight junction permeability and the structure and arrangement of the junctional fibrils has been reported in a recent freeze-fracture study of a number of epithelia. A direct correlation between the number of fibrils within the membrane and the degree of tight junction permeability was found (13).

The second approach is to find whether there are any detectable changes in the arrangement of the fibrils when the permeability of tight junctions is modified experimentally. One of the most striking cases of alteration of the permeability of the junctions is found when hypertonic solutions are added to the apical side of epithelia, such as those of the frog skin (14, 15) or the toad urinary bladder (16-20). The normally high transmural resistance of these epithelia markedly falls when hypertonic solutions are added to the apical side. Several lines of evidence show that this reduction in resistance is mainly due to the opening of a paracellular permeability pathway localized in the tight junctions (15-17, 19).

We describe here results obtained following both lines of inquiry. In one group of observations, we have compared the freeze-fracture morphology of the tight junctions in toad urinary bladder, a low permeability epithelium, with that of the rabbit ileum, an epithelium which according to both physiological (21) and morphological criteria (9) has permeable tight junctions (leaky junctions). In other experiments we have established that solutions made hypertonic with lysine induce a reversible opening of the tight junctions of the toad urinary bladder without gross deformation of the junction, as judged both by thin sections with electron-dense tracers and by freeze-fracture studies.

MATERIALS AND METHODS
Urinary hemibladders from toads, Bufo marinus, were mounted in normal Ringer’s solution for electrical measurements, as described (16).

The apical side was immersed in Tris/Ringer’s solution, where all the NaCl in the normal Ringer’s solution was substituted by Tris-chloride (pH 7.3-7.5). The fine structural distribution of lanthanum was studied as described (22). The distal 15 cm of the ileum was removed from rabbits anesthetized with Nembutal (40 mg/kg) and washed in cold Krebs’ solution equilibrated with 95% O2 and 5% CO2.

Samples for freeze-fracture were fixed during 1 hr in 2.5% glutaraldehyde, rinsed in 0.1 M cacodylate buffer, and infiltrated progressively with glycerol up to a 25% concentration. The tissues were frozen in Freon 22 and freeze-fractured at −115° in a Balzer’s 300 device. The replicas were observed with a Zeiss EM 10 electron microscope. The micrographs are mounted with shadow direction from the bottom; shadows are white.

To increase the number of tight junction fracture faces appearing in freeze-fracture replicas, we have used isolated epithelial sheets in some experiments. The methods used for separating epithelial sheets from the toad urinary bladder and the small intestine have been described (23-25). Careful comparison of replicas from whole tissues and isolated epithelial sheets, both of control and urea- or lysine-treated epithelia, revealed that the rapid obtention of epithelial sheets followed in a few seconds by fixation with glutaraldehyde did not induce changes in the structure of the tight junction.

RESULTS
Freeze-fracture morphology of tight junctions
Figs. 1 and 2 illustrate portions of tight junctions of the rabbit ileum mucosa and the toad urinary bladder epithelium,
respectively. Tight junction fibrils appear as ridges on the A or inner membrane face, and as grooves on the B or outer membrane face. Tight junctions of the rabbit ileum have a mean depth of 0.34 µm ± 0.01 SE (N = 55; range, 0.24–0.50 µm) and the mean number of fibrils was 6.72 ± 0.21 SE (N = 55; range, 5–10). In the toad urinary bladder, the mean depth of zonulae occludentes was 0.46 µm ± 0.02 SE (N = 19; range, 0.32–0.68 µm) and the mean number of fibrils was 8.7 ± 0.45 SE (N = 19; range, 6–12). Measurements were made as described earlier (13). In general, the extent of the junction exposed by the fracture process in epithelial cells is 2.0 µm or less in length. Therefore, it is not possible to rule out whether there are regions in the zonula occludens where the depth and the number of fibrils are different with respect to other regions examined in the replicas. However, on occasion the fracture plane exposed a large portion of the

FIGS. 1–3. Fracture faces of tight junctions in normal ileum epithelium (Figs. 1 and 3) and frog urinary bladder epithelium (Fig. 2). The membrane fibrillar network revealed by the fracture process tends to be similar in both epithelia. Figs. 1 and 2, ×55,800; Fig. 3, ×21,400.
remarkably different. The use of the basal molarity, it has been suggested that the opening of tight junctions of the toad urinary bladder caused by hypertonic solutions is necessarily associated with marked deformation of the junction (16, 17). We have surveyed the effects of a number of solutes on the resistance and morphology of this epithelium and have found that lysine causes an opening of the tight junctions with little structural deformation. 

Fig. 4 illustrates an experiment in which we measured the effects on the transepithelial resistance of progressively increasing concentrations of lysine in the apical solution. As with other hydrophilic solutes, lysine produces a decrease in transepithelial membrane resistance that was reversed when isotonic solution was replaced on the apical side of the epithelium. It was only necessary to increase osmolarity by raising the lysine concentration to 50-100 mM to observe a clear-cut reduction in resistance. When urea or sucrose is used, the minimal increases in osmolarity that produced a drop in transepithelial resistance corresponded to concentrations that varied between 100 and 150 mM for urea and 200 and 300 mM for sucrose.

To test whether the effect of lysine on transepithelial resistance was due to a chemical action or to the increase in osmolarity, we substituted isotonically Tris-HCl by lysine in some experiments and found that the transepithelial resistance was not altered by this procedure. In other experiments, solutions made hypertonic with lysine were placed on the basal side of the epithelium. When the concentration was increased up to 150 mM no effects were detected on transepithelial resistance. At 200 mM (or more) lysine in the serosal solution a reduction of transepithelial resistance was observed. These findings are similar to what is observed when other solutes, particularly sucrose and mannitol, are added to the serosal side of the toad urinary bladder (18).

Although the effects of lysine on the transepithelial resistance are similar to those observed when other solutes are used, the effect on the ultrastructure of the tight junctions is remarkably different. Examination of thin sections of toad urinary bladder epithelium fixed after 20 min of treatment with Ringer's solution ± 200 mM urea at the apical side revealed the presence of numerous large (0.2-1.0 μm) vesicles (17-19) within 50-80% of the tight junctions (Fig. 5). Examination of freeze-fracture replicas confirmed the existence of junctional blisters induced by hypertonic urea solutions and, in addition, demonstrated the disruption of the fibrillar network, particularly at the basal edge of the junction (Fig. 6). When the permeability of the tight junction in urea-treated urinary bladder was assessed by the addition of ionic lanthanum before fixation, it was found that a large percentage of the junctions, which in control epithelia are not permeated by lanthanum, were permeable to the tracer whether or not a vesicle appeared at the junction (19).

In sharp contradiction with the structural modifications induced by urea, the tight junctions of urinary bladders treated with solutions made hypertonic with lysine (100 or 120 mM) and examined in thin sections showed penetration of lanthanum into 90 to 95% of the junctions (Fig. 7) while less than 3% of the junctions examined revealed blisters. Tight junctions opened by lysine treatment (100 mM) showed a separation between adjacent membranes which varied between 3.0 and 12.0 nm in width. This opening of the tight junctions was reversible since in epithelia examined after return to isotonicity the junctions were impermeable to lanthanum, as in control samples. When the tonicity of the luminal solution was increased to 300 mM lysine, frequent blisters similar to those observed with urea were found. In freeze-fracture replicas of toad urinary bladders treated with 120 mM lysine for 20 min, the normal pattern of the junctional intramembrane fibrillar network is preserved (Figs. 8 and 9). In a total of 37 different junctions examined, blisters less than 0.15 μm in diameter were found only in one case. In the remaining junctions the number of fibrils, the depth of the junction, and the general structure of the fibrillar network remained similar to those seen in control epithelia. The mean number of junctional fibrils in bladders treated with 120 mM lysine was 8.2 ± 0.34 SE (N = 37; range, 5-13), and the mean depth of the junctions was 0.41 μm ± 0.12 SE (N = 37; range, 0.38-0.56 μm).

**DISCUSSION**

The first point to emerge from our results is that the relative permeability of tight junctions is not directly correlated with the number of junctional fibrils. It has been established by electron microscopic tracer studies (9, 17, 19) and electrophysiological techniques (21, 26) that the tight junctions of the rabbit ileum mucosa are of high permeability, while those of the toad urinary bladder are of low permeability. The electrical resistance of the paracellular pathway in the toad urinary bladder is at least of 12,000 Ω-cm² (26), while in the rabbit ileum the resistance is 125 Ω-cm² (21). The freeze-fracture data shown in this study demonstrate that the difference in the number of fibrils between these epithelia does not exceed 25%. If we assume that all the fibrils have uniform resistance properties, it is not possible to postulate that the differences in junctional permeability between rabbit ileum and toad urinary bladder arise solely from variations in the number of junctional fibrils. Further evidence in line with this conclusion arises from observations of the zonulae occludentes at the distal tubules of the rat kidney. This epithelium appears to have a relatively low transmural resistance (27) and its junctions are permeated by lanthanum (28, 29); however, freeze-fracture studies show a relatively
high number of intramembrane fibrils within the tight junctions (13, 30, 31). If the number of fibrils within the junctional membranes is not the sole determining factor of junctional permeability in epithelia, it is necessary to assume that some structural and/or chemical property of the junction, not revealed yet by freeze-fracture studies, is involved.

Further support for the notion that the sealing properties of the fibrils are not uniform under all circumstances comes from our experiments with lysine. This agent increased the permeability of the tight junctions of the urinary bladder without an obvious structural modification of the freeze-fracture morphology of the junctions. This finding is in contrast with the junctional opening induced by other solutes, which involves an extensive alteration of the fibrillar frame-work of the zonula occludens, as shown both by thin section (16, 17) and by freeze-fracture (32). Our results show again that a normal pattern of membrane fibrils of the tight junctions, as revealed by freeze-fracture, is compatible with a functionally leaky junction, as determined both by a decreased transepithelial electrical resistance and the penetration of lanthanum into the junction.

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