ROLE OF THE SEPTATE JUNCTION IN THE REGULATION OF PARACELLULAR TRANSEPITHELIAL FLOW

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The suggested classification of vertebrate epithelia as "tight" or "leaky" is based on the measured transmural conductance of these systems (10). The principal variation in conductance appears to be the degree of extra- or paracellular permeability rather than the permeability of cell membranes which are the principal resistive elements of the transcellular route for ion movement. Paracellular permeability, apparently not related to the active transport of ions, is largely limited by the "tight" junctions (8) which characteristically occur at the outward-facing or mucosal end of the epithelial intercellular space. Claude and Goodenough have shown that there may be a structural basis for variation in the intrinsic conductivity of these structures such that the number of appositional threads of "contact" between cells determines the magnitude of resistance provided by the junction (2). It is clear, nonetheless, that even very tight systems, like amphibian skin or urinary bladder with very restricted permeability to water and solutes, possess a finite degree of paracellular, "shunt" conductance (22, 3). A most provocative aspect of the junction in these systems is that junction conductivity, and hence paracellular permeability, is sensitive to the magnitude and direction of osmotic gradients applied across the epithelium. When these epithelia are exposed to osmotic gradients that favor water movement from the outside or mucosal surface to the serosa or blood side of the tissue, they allow only a trace absorption of water in the absence of antidiuretic hormone. If the gradient is reversed (i.e. favoring movement of water from serosa to mucosa), both the hydraulic and ionic conductivity of the paracellular pathway are strikingly increased (22, 21, 3). The osmotically induced increase in conductance is mediated by local accumulations of fluid, "blisters," in the intercellular space circumscribed by the contact zones of the tight junction (3, 24, 6, 23). This alteration of junction structure, first noted by electron microscopy, has been confirmed with in vitro examination of amphibian bladders by differential contrast microscopy (4). Identification of the junction as the site of changing conductance has also been demonstrated by cable analysis studies of this phenomenon (17). The relevance of these findings is that they have identified these rate-limiting junctions as structures with an intrinsic response to the osmotic changes encountered in the environment (5, 18). Thus, the shunting of spontaneous potential differences across epithelia will similarly be varied and, perhaps, in a manner that is essential to the efficiency or specificity of the transport function of the system.

The present study is, therefore, a first test of the hypothesis that epithelial function depends to some extent on the presence of an osmotically adjustable, paracellular shunt pathway. Our rationale is that if such a dependence exists, it would very likely apply to invertebrate epithelia which certainly comprise the functional analogues of their vertebrate counterparts and where selection pressures on the evolution of epithelial design may thus be presumed to have been identical. More specifically, we have asked whether the structure of invertebrate septate junctions possesses an osmotic sensitivity similar to that of vertebrate tight junctions, since these structurally different cell contacts should be functionally analagous by the reasoning above. The surface epithelium of the

freshwater planarian, *Dugesia tigrina*, has been initially chosen as an example of a readily studied invertebrate system with an obvious ability to resist the strong, ambient osmotic forces which would otherwise swell the organism with water from the very dilute external environment.

MATERIALS AND METHODS

Specimens of the planarian, *D. tigrina*, were obtained from Connecticut Valley Biological Supply (Southampton, Mass.) and maintained in aerated Connecticut Valley springwater. Animals were fed fresh rat liver once a week but were starved for 1 wk before experimentation.

The experimental protocol involved immersion of whole organisms in either unaltered springwater (pH 7.54, 2–5 mosmol/kg H_2O) or in this medium after elevation of osmolality to a prescribed level with either NaCl, KCl, urea, mannitol, or sucrose. All experiments were performed at room temperature (23–25°C) and with continuous aeration of solutions.

Specimens for electron microscopy were fixed after 10 min of exposure to the medium by the addition of 50% (wt/vol) glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) to a final concentration of 1.0%, and allowed to stand 10 min with continued aeration before transfer to 1% glutaraldehyde in phosphate buffer for overnight fixation at 4°C. Subsequent processing involved a 30-min rinse in the buffered vehicle, 30 min in 1% OsO_4 in the same buffer, dehydration in ethanol, and embedment in an Epon-Araldite mixture as previously described (7). One-half of each sample was stained, en bloc, with uranyl acetate. Thin sections were cut with an LKB Ultrotome III ultramicrotome (LKB Produkter, Bromma, Sweden) from blocks selected at random from both dorsal and ventral surfaces throughout the length of the body. Sections were stained with uranyl acetate and lead citrate (19) and examined in a Philips EM 200 electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, N. Y.). A portion of the study involved coding of sample sections to preclude interpretive bias.

To employ the planarian epidermis as the material for this study, it was specifically necessary to verify the assumption that the osmolatity of intercellular fluid in *D. tigrina* exceeds that of the animal's natural environment (16).

Organisms were briefly blotted, weighed in tared vials, dried out at 70°C for 16–20 h, and reweighed in vials to obtain wet weight/dry weight ratios. Dried specimens were macerated in small volumes of distilled, deionized water to dissolve total ions so that Na^+ and K^+ concentrations could be determined by flame photometry and so that osmolality could be measured. Assuming identical osmolalities for intra- and extracellular fluids, estimates of minimal intercellular fluid osmolality were thus obtained. A more precise determination (with suitable corrections for fluid compartmentalization) was beyond the scope of the present study.

RESULTS AND DISCUSSION

Epidermal cells of the freshwater planarian, *D. tigrina*, although of several different types (13), are uniformly joined by septate junctions at the apical, or outward-facing, end of the epithelial intercellular space. When organisms were fixed after being exposed only to springwater, the appearance of these structures was similar to that described for the junctions of several other invertebrate epithelia. In thin section view (Fig. 1 *a*), the planarian septate junction is a region where adjacent cell membranes are roughly parallel and separated by 17–18 nm; the intercellular space is bridged by as many as 20 dense bands of 5 nm thickness. These bands are irregularly spaced with an average separation of 10 nm. No significant variations of this structure were observed in the course of examining multiple sections from each of six organisms fixed after exposure to only unaltered springwater. In every instance, the observed junction profiles comprised a series of rectilinear spaces bounded by adjacent septae and cell membranes. While the designation "septate junction" or "septate desmosome" has been rather broadly applied, it is now clear that at least two general forms are found. It has been suggested that these be specified as "pleated" or "continuous" to more accurately described the conformation of the individual septae which are arranged in roughly parallel fashion traversing the intercellular space (9). Planarian junctions are, by this classification, probably best described as pleated septate junctions (with septae in the "chair" conformation) since they give rise to a readily discernible ladder-like array in thin section views (Fig. 1 *a*). In this respect they are similar to the junctions of *Hydra* epidermis (12) rather than to the junctions in organisms of higher invertebrate phyla.

Determination of intercellular fluid osmolality yielded the following results. Organisms had wet:dry weight ratios of 5:1; mean osmolalities of 110 mosmol/kg H_2O; Na^+ and K^+ concentrations of 16 and 36 mM, respectively. Assuming that cells of this organism are roughly isotonic to the intercellular fluid and that intracavitary water remaining after blotting might be as little as 10% of total body water, a lower limit of approximately 120 mosmol/kg H_2O was taken as appropriate for intercellular fluid osmolality. Given the possibility for substantial experimental error, it is nonetheless evident that the organism is decidedly hypertonic to the <6 mosmol fluid of its natural environment.

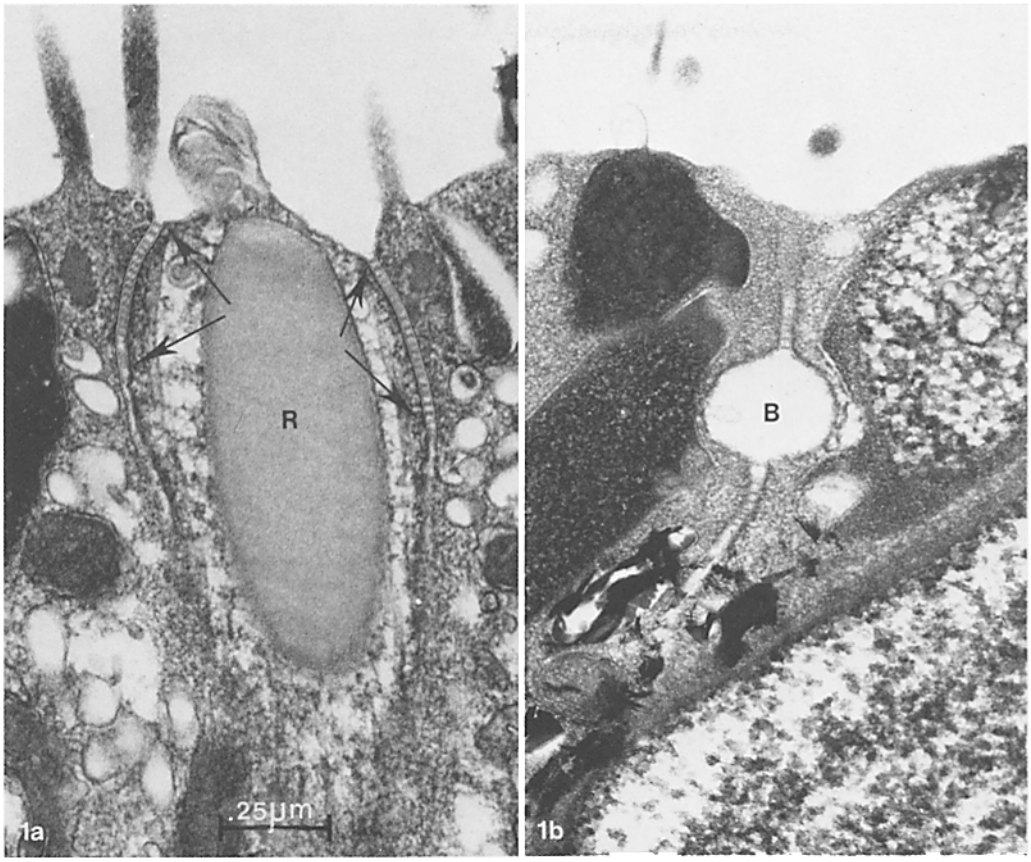

FIGURE 1 (*a*) View of planarian epidermis showing septate junctions (extended between unlabeled arrows). In specimens fixed by addition of glutaraldehyde to unaltered springwater, these structures appear as shown. Septal spacing and length of the junction itself is variable, but bullous distensions (as in *b*) were not found in any of the numerous profiles examined from each of several specimens. The junctions shown bound a rhabdite-bearing cell with a typical inclusion (*R*). × 60,000. (*b*) Septate junction fixed after a 10-min exposure to 400 mM mannitol in spring water. The profile of a fluid blister (*B*) shows a mean diameter of about 0.25 μm × 60,000.

No changes in junction structure were detected when organisms were fixed 10 min after elevation of medium osmolality to 150 mosmol/kg H_2O with either NaCl, KCl, urea, mannitol, or sucrose. Further, there was no observed change in the epithelial cells in terms of vacuolation or apparent volume, although the possibility of minor changes in these features was not pursued in detail. This level of environment tonicity is, by our estimation, close to isotonic with the intracellular space so that very little gradient of water activity is established across the junction; it is thus unlikely that any osmotic flow-dependent structural alteration of the junction would be produced under these conditions.

However, progressive additional elevation of osmolality was found to effectively distort or "blister" the space between septae in a manner that was dependent upon choice of solute. Fig. 1 *b* depicts the type of distortion noted. Threshold concentration, the level at which a "blistering" was readily observed, was roughly proportional to the molecular weight of the solute used. NaCl, (mol wt 58.44), KCl, (mol wt 74.56), and urea (mol wt 60.06) produced blistered profiles when employed at a level of 300 mosmol/kg H_2O. With mannitol (mol wt 182), 400 mosmol/kg was required to elicit the phenomenon, and with sucrose (mol wt 360), 600 mosmol/kg was required. Production of the phenomenon and the variety of observed junction profiles are illustrated in Fig. 2 *a–c*. A relationship of this sort is best explained if the phenomenon is assumed to be dependent

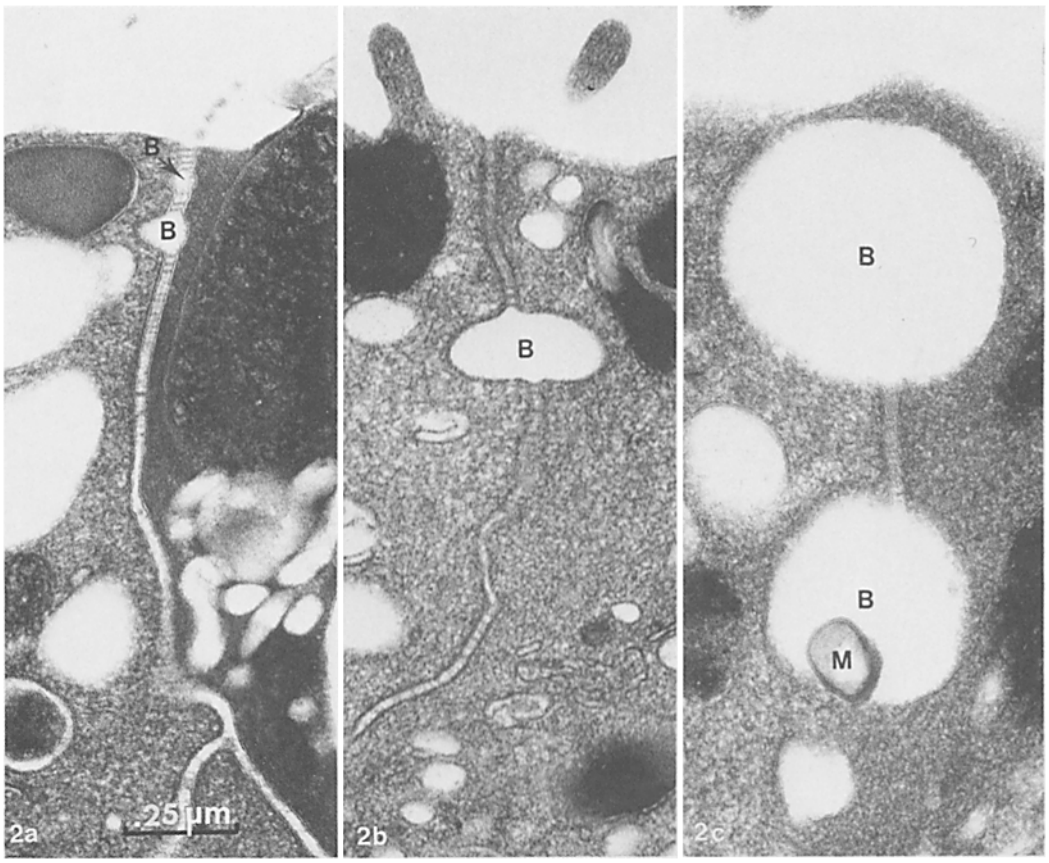

FIGURE 2 (*a*) Specimens treated with 400 mM mannitol for 10 min before fixation. The blisters (*B*) in this junction profile are among the smallest observed. × 60,000. (*b*) Blistered junction profile from specimens fixed 10 min after immersion of organism in medium brought to 300 mosmol/kg H_2O with KCl. × 60,000. (*c*) Large fluid blisters (*B*) after treatment with 400 mM mannitol. The lower distension contains a "myelin" figure; similar membranous inclusions have been noted in the comparable phenomenon in toad bladder (2). × 60,000.

upon solute permeation of the junction, as has been proposed for the comparable phenomenon in toad bladder (3, 24, 1, 20) and skin (3).

Two aspects of this finding required verification beyond a purely subjective analysis. The presence of blistered junctions was tested only in samples where the outside medium was hypertonic to intercellular fluids. These sections were labeled in coded fashion for examination by one of us without prior knowledge and sample source. A single section was prepared from each of two previously unexamined tissue blocks from each of three experimental groups: tissues exposed to 300 mM urea, tissues exposed to 400 mM mannitol, or untreated tissues. All epidermal junctions found were classified as blistered or normal for each section. Decoding eventual results revealed that the fraction of blistered junctions were as follows: 32/46 and 46/57 for urea, 31/52 and 21/44 for mannitol, and 0/42 and 0/37 for untreated tissue. It was clear that junction distortion was systematically related to the application of a hypertonic external medium, which was in agreement with the initial studies using three animals from each of these protocols.

Coded labeling of additional material was also employed as a means of comparing the relative effectiveness of solutes (i.e., urea, KCl, mannitol, and sucrose). When these specimens were ranked subjectively, on the basis of the observed degree of blistering, the effects of urea (at 300 mM) and KCl (at 150 mM) could not be distinguished from

each other, but each led to a substantially greater frequency of blistering than mannitol (at 400 mM) or sucrose (at 600 mM) which were similarly indistinguishable. In this way we were assured that the effect was inversely dependent on the amount of the solute used to render the solution hypertonic, as was suggested above with determination of the threshold or minimum level of solute concentration required for production of detectable junction distortion. In each case, demonstration of a permeation-dependent osmotic phenomenon points to the existence of a finite permeability to small hydrophilic molecules, water, and, most importantly, to the principle ions (Na^+, K^+, Cl^-) of body fluids and the environment.

Rigorous quantitation of the actual frequency of distorted junctions did not seem worthwhile since we were technically unable to correlate such morphological evidence for increased paracellular permeability with any electrical or chemical index of this parameter.

These observations support the hypothesis that the invertebrate septate junction is functionally analogous to the tight junction of vertebrate systems. On purely structural grounds, each junction has often been presumed to constitute a significant barrier to paracellular flow (e.g., references 11 and 14). The fact that each is structurally sensitive to transepithelial osmotic gradients has additional bearing on their probable roles in the transport function of epithelia. While these rate-limiting barriers to paracellular flow are strikingly different in their overall morphology, each seems subject to the same fundamental considerations in design. In each case a serial arrangement of discreet and finitely permeable barriers is arranged so that a portion of the lateral intercellular space is divided into a number of fairly discreet compartments. Solute and/or solvent access to these compartments differs significantly depending on the side of the epithelium from which penetration is attempted. Effectively, the junction defines a region of lateral intercellular space that is bounded by asymmetric barriers and is thus obliged to constitute a rectifying element within the paracellular pathway (20). (The thermodynamic explanation for this obligatory rectification by a pair of dissimilar barriers in series has been provided by Patlak et al. [15].) Since the compartments so delineated in both vertebrate and invertebrate junctions are also distensible (swelling with internal hydrostatic pressure from fluid accumulation), it is apparent that the intrinsic conductivity of the junction may be adjusted by a modulation of the width of the intercellular space within the confines of this rate-limiting barrier. Coupling this feature with the generally accepted conclusion that the paracellular pathway is the route of counter-ion movement for active transepithelial transport (e.g. Cl^- in the case of Na^+ transport by the toad bladder), one is tempted to postulate that these junctions are ideally constructed to function as "feedback" elements in the regulation of transepithelial active transport, although detailed speculation on this point seems unwarranted. Yet, the striking parallelism in structural responsiveness to transepithelial osmotic gradients cannot be dismissed as coincidental but rather may be viewed as indicative of some selected "functional" characteristic.

It seems safe to conclude that both "tight" and "septate" junctions constitute the rate-limiting barriers to the passive, paracellular flow of water and small molecules in the epithelia where they reside. Each might be functionally classified as a "limiting junction" as suggested earlier for the vertebrate variety (3 and 6). A working hypothesis is proposed that "tight" and "septate" junctions share a purposeful, intrinsic response to ambient levels of tonicity or water activity so that an epithelium's resistance to paracellular flow is modulated with changes in its fluid environment.

SUMMARY

A comparison of the distribution of septate junctions in invertebrate epithelia and tight junctions in vertebrate systems suggests that these structures may be functionally analogous. This proposition is supported by the internal design of each junction which constitutes a serial arrangement of structures crossing the intercellular space between cells to effectively provide resistance to the paracellular flow of water and small molecules. We have tested the validity of such an analogy by examining whether the osmotic sensitivity of the septate junctions of planarian epidermis follow the rather striking pattern observed for the junctions of very tight vertebrate epithelia (e.g. toad urinary bladder). It has been found that the septate junctions in this system respond in similar fashion to their vertebrate counterparts, blistering with accumulated fluid when the medium outside the epidermis is made hypertonic with small, water-soluble molecules. We conclude that the two types of junction probably are functionally analogous and that, in each case, this rectified structural response to transepithelial osmotic gradients may be indica-

tive of the role of such structures in the transport function of epithelia.

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