Differential regulation of tight junction permeability during development of the retinal pigment epithelium

YURIKO BAN AND LAWRENCE J. RIZZOLO

Departments of Surgery and of Ophthalmology and Visual Science, Yale University, New Haven, Connecticut 06520-8062

Received 20 October 1999; accepted in final form 24 March 2000

Ban, Yuriko, and Lawrence J. Rizzolo. Differential regulation of tight junction permeability during development of the retinal pigment epithelium. Am J Physiol Cell Physiol 279: C744–C750, 2000.—The retinal pigment epithelium (RPE) is an epithelial region of the blood-brain barrier. During embryogenesis, permeability of the barrier gradually decreases. A culture model of RPE development revealed differences in how tight junctions regulate the paracellular diffusion of ionic and nonionic solutes (Ban Y and Rizzolo LJ. Mol Vis 3: 18, 1997). To examine these differences, the permeation of ionic and nonionic monosaccharides was compared with mannitol, and the permeation of the alkali metals was compared with sodium. The order of permeation was 3-O-methylglucose = glucosamine = mannitol > N-acetylneuraminic acid. The ratio of N-acetylneuraminic acid to mannitol permeability decreased with embryonic age of the RPE or exposure to retinal-conditioned medium. Neither the ratio nor the permeability was affected by inhibiting transcytosis. The ratio increased if tight junctions were disrupted in low-calcium medium. The permeation of cations followed the sequence cesium > rubidium > potassium = sodium > lithium and was unaffected by embryonic age or retinal-conditioned medium. These results are considered in terms of a model in which the size distribution, charge, or number of open junctional pores could be modulated. It suggests that different subpopulations of pores can be regulated independently during development.

blood-retinal barrier; paracellular permeability; epithelium; intercellular junction; retina

ORIGINALLY, TIGHT JUNCTIONS were defined as an occluding seal between the neighboring cells of an epithelium. This seal encircled each cell to retard diffusion through the paracellular spaces and allowed the epithelial cells to regulate transport between compartments of different composition. It is now understood that the flux across tight junctions can be large and that the permeability and selectivity of the junctions can be regulated (5). Besides tight junctions, the paracellular space can also contribute to barrier function (6). Often this contribution is small, but it can become significant when the width of the paracellular space is <10–20 nm.

At tight junctions, the neighboring plasma membranes closely appose or “kiss.” With freeze-fracture microscopy, these kisses appear as a series of continuous, anastomosing strands that encircle the cell. It is commonly postulated that pores in these strands permit the paracellular flux of solutes. The observation that permeability can decrease exponentially with an increase in strand number suggests that these pores fluctuate between open and closed states (2, 6, 16). This mechanism has been invoked to explain how overexpression of the tight junction protein occludin can lead to an increase in the permeability of mannitol but a decrease in the permeability to ions (2, 18). These authors reasoned that the apparent discrepancy resulted from the methods used to measure permeability. Permeability to ions is inversely proportional to the transepithelial electrical resistance (TER). Because this is an instantaneous measurement, pores in each strand must be open simultaneously for current to pass. Permeability to mannitol is measured by tracer diffusion. This slow measurement is less sensitive to the open-closed state of the pores.

In the epithelia and endothelia that form the blood-brain and blood-retinal barriers, the functional properties of tight junctions develop gradually during embryonic development (8, 23, 27). Therefore, development can be used to dissect function into component parts. A convenient model to study this process is the chick retinal pigment epithelium (RPE). The RPE forms the outer blood-retinal barrier by separating the photoreceptors from fenestrated capillaries in the choroid (17). The gradual development of RPE tight junctions can be divided into early, intermediate, and late phases (23). Early junctions express occludin and ZO-1, but are leaky. During the intermediate phase (embryonic days 7–15, E7–E15), there are changes in the expression of ZO-1, and the permeability to horseradish peroxidase (HRP) decreases (35). Nonetheless, there is little change in number or complexity of tight junctional strands until the late phase (14). We devised a novel culture model to study the changes in permeability that occur during the intermediate phase (3, 24).

This culture model permits further examination of the pore concept. RPE was isolated from E7, E10, and E14 embryos and cultured on laminin-coated filters.
During development, permeability to ions and nonionic solutes appeared to be regulated by independent mechanisms. These changes in permeability resulted from a normal, developmental process.

In the current study, we used two methods to examine the role of size and charge on tight junction selectivity. We compared permeation of charged and uncharged solutes with a hydrodynamic radius close to 4–5 Å and determined the relative permeation of the alkali I series of cations. The data are discussed in light of a model in which pores can vary in size, and the distribution of pore sizes can be shifted to semiselectively regulate paracellular diffusion.

**MATERIALS AND METHODS**

**Cell culture.** RPE was isolated from E7, E10, and E14 embryos of white Leghorn chickens and was cultured on 12-mm Transwell filters (Corning Costar, Cambridge, MA). The filters were freshly coated with laminin (5–10 μg/cm²; UBI, Lake Placid, NY). There was variability among lots of laminin. By increasing the amount of laminin on the filter, TER of the cultures increased until a plateau was reached. This maximal value was fairly constant and depended only on the embryonic age of the RPE. For consistency, the amount of laminin used was the minimum needed to reach this plateau. Cultures were maintained in serum-free medium (SF2; see Ref. 3). For some cultures, the medium in the apical medium chamber was replaced with SF2 that was conditioned by E14 retinas. Again, the effect on ions was greater than the effects on mannitol. During development, the permeability to ions and nonionic solutes appeared to be regulated by independent mechanisms. These changes in permeability resulted from a normal, developmental process.

To measure transmonolayer diffusion in low-calcium medium, cultures were maintained in SF2 or retinal-conditioned medium for 9 days and were switched to DMEM containing 5 μM calcium for 15 min at 37°C. Radiolabel was added to this medium and incubated as described above. Aliquots of 5 μl were taken from both chambers at 5 h.

To determine ion selectivity, the TER was measured in 20 mM HEPES-buffered DMEM (pH 7.0), as described previously (3). These electrodes give a uniform field distribution across the filter and consequently are not subjected to the artifacts associated with chop-stick electrodes. They are well suited to measure TER in cultures of high permeability.

To determine ion selectivity, the TER was measured in 20 mM HEPES buffer (pH 7.0) containing 3 mM CaCl₂, 10 mM glucose, 1 mM sodium pyruvate, and 165 mM chloride salt of either lithium, sodium, potassium, rubidium, or cesium. The osmolality of the solutions (333 mosmol/kgH₂O) was verified with an Advanced Laboratory wide-range osmometer (Advanced Instrument, Needham Heights, MA). The TER was measured in HEPES-buffered DMEM before and after each buffer change to verify that the monolayers were unaltered during the experiment. Two buffers could be reliably tested on each filter. Measurements were always made with sodium chloride and one other salt. Filters were washed three times for each buffer change. Permeation is proportional to the reciprocal of the TER. Therefore, permeation(\(X_{\text{permeation(sodium)}}\)/TER(sodium))/TER(X) = \(P\) where \(\frac{X_{\text{permeation(sodium)}}}{\text{TER(sodium)}}\) is the permeation of mannitol in the basal chamber; \(X_{\text{permeation(sodium)}}\) is the initial concentration in the apical chamber, \(A\) is the area of the filter in cm², and \(T\) is the time in minutes. The final units become microliters per square centimeter per minute. \(P\) was calculated as the slope of the linear regression line of \(\frac{X_{\text{permeation(sodium)}}}{\text{TER(sodium)}}/\text{cm}^2\) vs. time.

As indicated in Table 2, some cultures contained 50 μM chloroquine (Sigma) or were incubated at 4°C. With low temperature incubation, aliquots of 5 μl were taken from both chambers at 5 h.

Results

Mannitol and several monosaccharides were selected for this study because they would be of similar size (hydrodynamic radius close to 4–5 Å) but would vary in charge (28). Mannitol is commonly used to measure at 30, 60, and 90 min. Radiolabeled tracers were quantified by counting aliquots in a liquid scintillation counter (LKB Instruments, Gaithersburg, MD). The flux was linear during the experiment, as reported in previous studies (3). Nonlinear data were excluded from the analysis. The flux depends on the initial concentration of solute in the apical chamber. To normalize the data, the permeability \(P\) was calculated by the following formula (25, 26, 33):

\[
P = \frac{X_B}{X_{\text{B0}}/A/\text{cm}^2}
\]

where \(X_B\) is counts per minute in the basal chamber; \(X_{\text{B0}}\) is the initial concentration in the apical chamber, \(A\) is the area of the filter in cm², and \(T\) is the time in minutes.
transepithelial diffusion across the paracellular space because epithelial transporters for mannitol have not been described (16). Vertebrate transporters for unmodified N-acetylneuraminic acid have only been described in lysosomes (11). However, glucose transporters in RPE can facilitate the transcellular diffusion of glucosamine and 3-O-methylglucose. This transcellular pathway was inhibited by a combination of high glucose and phloretin (21). Phloretin did not affect the paracellular pathway in the gallbladder (33). In RPE, phloretin reduced transepithelial diffusion of glucosamine and 3-O-methylglucose to the level of mannitol without affecting the diffusion of mannitol or N-acetylneuraminic acid (Ban and Rizzolo, unpublished observations and Ref. 4).

To determine whether ionic charge affected the permeability to monosaccharides, the permeation of each monosaccharide was simultaneously measured with mannitol in the same culture. The permeation of mannitol was the same as the permeation of the neutral 3-O-methylglucose and the cationic glucosamine (Fig. 1). This was also observed when the permeability of the cultures was reduced by E14 retinal-conditioned medium and when RPE was isolated from embryos of various ages. By contrast, the permeation of N-acetyl-

neuraminic acid was lower than mannitol for all cultures. The errors in these measurements include the variability in permeability among different preparations of RPE. This source of error can be eliminated by determining the monosaccharide-to-mannitol ratio for each individual culture and averaging the ratio (Table 1).

The N-acetylneuraminic acid-to-mannitol ratio varied with culture conditions (Table 1). Consistent with Fig. 1, the ratio of other monosaccharides to mannitol was close to 1.0 in all circumstances. There was no statistical difference in the N-acetylneuraminic acid-to-mannitol ratio between E7 and E10 cultures, but retinal-conditioned medium decreased the ratio to the level of E14 cultures ($P < 0.05$). The ratio in E14 cultures was unaffected by conditioned medium.

These data confirm previous studies that the TER increases out of proportion to the decreases in mannitol permeability. Furthermore, there was no correspondence between changes in the relative permeability to N-acetylneuraminic acid and the TER. The permeability to mannitol decreased 38% between E7 and E10, but the permeability to ions decreased 54% (Figs. 1 and 2). A similar result was obtained with cultures maintained in retinal-conditioned medium. This is striking because when the TER is $<200 \, \Omega/cm^2$ a small change in TER is usually accompanied by a large change in the permeability to mannitol (16). Despite the change in TER between E7 and E10, the ratio of N-acetylneuraminic acid to mannitol was unchanged. Similarly, the ratio was unchanged despite a large change in TER between E14 cultures with or without conditioned medium.

The change in ratio could result from changes in the negative charge density of surface glycoproteins. For example, repulsion by surface charge might retard the entry of N-acetylneuraminic acid in transcytotic vesicles or selectively reduce the width of the channels between the lateral membranes of neighboring cells. To inhibit or retard vesicular traffic, the cultures were incubated at 4°C or in the presence of chloroquine (1, 15). Neither treatment affected the N-acetylneuraminic acid-to-mannitol ratio (Table 2).

To distinguish between an effect of tight junctions from an effect of the lateral space, we exploited an

<table>
<thead>
<tr>
<th>Table 1. Ratio of monosaccharide-to-mannitol permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>3-O-methylglucose</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Glucosamine</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>N-acetylneuraminic acid</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SE of 6 experiments. E7, E10, and E14, embryonic days 7, 10, and 14, respectively; SF2, serum-free medium; CM, conditioned medium.
unusual feature of chick RPE. Standard procedures for isolating chick RPE rely on an adhesion mechanism that is calcium independent. Sheets of RPE are released from the basement membrane by calcium chelators in the absence of proteases (12, 34). Single cells are then obtained by using trypsin to disrupt interactions between the lateral membranes. In our experiments, media that lacked calcium disrupted tight junctions, but the lateral membranes remained adherent near the apical end. Examples are shown in Fig. 3. E7 and E14 cultures were maintained in retinal-conditioned medium for 9 days and then were incubated for 1 h in DMEM containing 5 \( \mu \text{M} \) calcium. In each case, the TER was reduced to 8–10 \( \Omega \cdot \text{cm}^2 \) without affecting the cuboidal morphology of the monolayer. The electron micrograph reveals adhesions along the lateral membrane that were confined to the apical end. The confinement of lateral junctions to an apical junctional complex is typical of RPE in vivo and in normal culture conditions (13, 22, 23). Similar results were obtained with cultures maintained in SF2 except that punctate microwounds developed where RPE came off the filter. To look for microwounds in the conditioned medium-treated cultures, the cultures were stained with phalloidin to reveal the actin cytoskeleton. No wounds were evident by this procedure (data not shown). Therefore, cultures maintained in conditioned medium were used.

Table 2. Ratio of N-acetylneuraminic acid-to-mannitol permeability in E7 and E14 cultures that were incubated with chloroquine or at 4°C

<table>
<thead>
<tr>
<th></th>
<th>E7</th>
<th>E10</th>
<th>E14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SF2</td>
<td>CM</td>
<td>SF2</td>
</tr>
<tr>
<td>Chloroquine (50 ( \mu \text{M} ))</td>
<td>0.75 ± 0.02</td>
<td>0.68 ± 0.03</td>
<td>0.65 ± 0.01</td>
</tr>
<tr>
<td>4°C</td>
<td>0.76 ± 0.03</td>
<td>0.68 ± 0.02</td>
<td>0.66 ± 0.01</td>
</tr>
</tbody>
</table>

Data are means ± SE of 3 experiments.

Table 3. Ratio of N-acetylneuraminic acid-to-mannitol permeability in 5 \( \mu \text{M} \) Ca\(^{2+}\)

<table>
<thead>
<tr>
<th></th>
<th>E7</th>
<th>E10</th>
<th>E14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(( \mu \text{l/cm}^2\text{min} ))</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.84 ± 0.08</td>
<td>0.92 ± 0.14</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>( P_{\text{mannitol}} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.81 ± 0.02</td>
<td>0.76 ± 0.01</td>
<td>0.76 ± 0.01</td>
</tr>
</tbody>
</table>

Data are means ± SE of 3–7 experiments. \( P_{\text{mannitol}} \), mannitol permeability; \( P_{\text{N-acetylneuraminic acid}}/P_{\text{mannitol}} \), N-acetylneuraminic acid permeability. E14 cultures were maintained in retinal-conditioned medium and transferred to low-calcium medium.
to measure the N-acetylneuraminic acid-to-mannitol ratio. In each culture, the ratio increased, indicating that the permeation of the anionic solute increased more than the similar-sized nonionic solute (Table 3). This suggests that pores contribute to the N-acetylneuraminic acid-to-mannitol ratio.

Tight junctions can exhibit a slight cation selectivity that varies among epithelia (20). To determine if cation selectivity changes with age or in response to conditioned medium, the TER was measured in HEPES-buffered saline in which the cation was varied. After each measurement, the TER was measured in HEPES-buffered DMEM to verify that the monolayer was unaltered by the change in buffers. The ratio of the TER in sodium salt to the TER in the test salt equals the permeability ratio between the test cation and sodium (Table 4). These data demonstrate that the relative permeability of ions was cesium > rubidium > sodium, potassium > lithium. This conforms most closely to the Eisenman sequence I for channels of low ionic field strength (10). This selectivity was not altered by conditioned medium and was unaffected by age.

### DISCUSSION

The results of this study were unaffected by treatments that inhibit transcytosis but were affected by treatments that disrupt tight junctions. Therefore, these data were considered in terms of effects on tight junctions or on the lateral spaces. A common model for tight junctions is a network of anastomosing strands that contain pores whose open-closed state can be regulated. A variation of this model is presented in Fig. 4.

Pores that vary in size and charge are shown. These may be viewed as the total number of pores or the number of pores in their open state. Because this model is concerned with the intermediate stage of development (see introduction), the number of strands and their complexity are held constant.

**Mechanism A** (Fig. 4) shows a shift in size distribution. The loss of large pores is compensated by an increase in small pores. The total cross-sectional area of pores remains unchanged. With this mechanism, larger solutes would be excluded but the permeation of smaller solutes would be unaffected. **Mechanism A** could account for the differences between E7 and E10 RPE that were cultured in SF2. Previous studies showed that the permeability to HRP decreased without a corresponding decrease in the smaller solutes inulin and mannitol (3). Permeation might be affected by the charge on the walls of the pores if the predominant pores were close in size to the solute. For example, between E10 and E14 there is a decrease in the permeation of inulin and N-acetylneuraminic acid, but there is less of an effect on mannitol (Table 1; see Ref. 3).

**Mechanism B** (Fig. 4) shows a decrease in the number of pores (or time in the open state) without affecting the size distribution. This would cause a decrease in the permeability of all solutes, without an effect on charge selectivity. This mechanism could explain the effects of retinal-conditioned medium on the E14 cultures. A variation on this mechanism is to preferentially decrease the number of large pores. In this scenario, the total cross-sectional area decreased, and the effects on charge discrimination by smaller pores would become more evident. This might explain the effect of retinal-conditioned medium on the E7 and E10 cultures. Conditioned medium reduced the permeability of all solutes, but N-acetylneuraminic acid was affected more than mannitol.

The analysis thus far applies to pores with a radius >5 Å. Smaller pores, but large enough for ions, would be invisible to the solutes mentioned above. A decrease in the number of such pores would explain an increase in TER that was out of proportion with a decrease in

### Table 4. Ratio of alkali metal-to-sodium permeability

<table>
<thead>
<tr>
<th></th>
<th>Cesium/Sodium</th>
<th>Rubidium/Sodium</th>
<th>Potassium/Sodium</th>
<th>Lithium/Sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7 SF2</td>
<td>1.44 ± 0.10</td>
<td>1.15 ± 0.04</td>
<td>1.02 ± 0.05</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>E7 CM</td>
<td>1.33 ± 0.02</td>
<td>1.09 ± 0.02</td>
<td>1.09 ± 0.10</td>
<td>0.81 ± 0.05</td>
</tr>
<tr>
<td>E14 SF2</td>
<td>1.40 ± 0.04</td>
<td>1.15 ± 0.05</td>
<td>1.03 ± 0.12</td>
<td>0.82 ± 0.07</td>
</tr>
<tr>
<td>E14 CM</td>
<td>1.38 ± 0.06</td>
<td>1.24 ± 0.03</td>
<td>1.17 ± 0.12</td>
<td>0.88 ± 0.05</td>
</tr>
</tbody>
</table>

Data are means ± SE of 4–10 experiments.
mannitol permeability. This was observed among cultures of different age that were maintained in either SF2 or retinal-conditioned medium. According to studies on other epithelia, the relationship between mannitol permeation and TER is nonlinear (16). When the TER is $<200 \ \Omega \cdot \text{cm}^2$, a decrease in junction permeability affects mannitol more than ions. In contrast, the percent increase in TER that we observed was always greater than, or equal to, the percent decrease in permeability to mannitol.

Mechanism C (Fig. 4) shows a change in the charge density of the pores. This would be a nonexclusive, alternative explanation for the decreased N-acetylneuraminic acid-to-mannitol ratio. However, it would not explain the concomitant changes in mannitol permeation or size discrimination. We looked for other evidence of this mechanism by determining ion selectivity. Among the various epithelia, the most selective is the gallbladder. Depending on the species, ion selectivity conforms to the Eisenman sequence II or IV (10, 19, 20). These sequences correspond to channels of low or moderate “field strength.” The ion selectivity of RPE junctions always conformed to sequence I, which corresponds to channels of lowest field strength. Either the selectivity of the pores is low or the selectivity of small pores is masked by the flux through large, nonselective pores. The latter explanation, combined with variation in the distribution of pore size, could explain the variation in ion selectivity among various epithelia.

A variation of mechanism C would be a change in the charge of the lateral membranes. For example, charge density would increase if the content of sialic acid increased in the membrane glycoproteins near the apical junctional complex. The apical junctional complex of RPE resembles the junctional complex of endothelial cells, where tight junctions are intermixed with gap and adheren junctions (13). Because very few other junctions exist along the remainder of the lateral membranes, neighboring lateral membranes are close together only for a short distance at their apical end. Because of the presence of calcium-independent adhesion (12, 34), this close relationship was maintained when low-calcium medium was used to disrupt tight junctions (Fig. 3). There was no obvious change in the lateral space observed by transmission electron microscopy. This may explain why the permeability to mannitol failed to rise more than twofold, even when the junctions were disrupted in low-calcium medium. An increase of negative charge on the membranes might affect N-acetylneuraminic acid more than mannitol, thereby restricting access of N-acetylneuraminic acid to the pores. The change in ratio in response to low-calcium medium was rapid and was unlikely to be due to the turnover of glycoproteins. Conceivably, low-calcium medium causes the selective endocytosis of sialylated glycoproteins, but the simplest explanation is an effect on tight junctional pores.

The model presented in Fig. 4 was developed because models of uniform pores were unable to explain the data from this and our earlier studies. This model is practical because of the molecular complexity of the tight junctions (32). The scaffold of the junction is formed by three members of the membrane-associated guanylate kinase homologues protein family (9). This family assembles signaling, receptor, and transport complexes at a variety of membrane junctions. In the tight junction, family members ZO-1, ZO-2, and ZO-3 each contain a series of protein-binding domains, including three postsynaptic density protein-95, discs large tumor suppressor A, zona occludins-1 (PDZ) domains, an Src homology 3, and a guanylate kinase-like domain. Despite the similarity of the amino acid sequence of the PDZ domains, the differences are highly conserved between avian and mammalian species (7). This suggests that a complex of the three proteins can assemble a wide variety of structural and regulatory proteins. Several kinases and G proteins concentrate at the tight junctions along with two types of transmembrane proteins. The first of these, the claudins, are transmembrane proteins that form the strands of the tight junction (30). Sixteen claudins have been identified, and these are the only junctional proteins known to have tissue specificity (32). One of these, claudin 16 (paracellin-1), participates in a magnesium-specific channel of renal tight junctions (29). The second known transmembrane protein of the junctions, occludin, is a regulatory protein that is controlled by phosphorylation (18, 31). By incorporating different mixtures of claudins and occludin, pores of varying size and charge properties could conceivably be assembled. A flexible, regulatable scaffold could contribute to diverse pore structures. The developmental model described here will permit a more-detailed examination of this hypothesis.

We thank Eric Reardon for expert technical assistance and Steven Wilt for critically reviewing the manuscript.

This work was supported by National Eye Institute (NEI) research grant EY-08694 (L. J. Rizzolo), Association for Research in Vision and Ophthalmology/Alcon Research and Judson-Coxe Fellowship Grants (Y. Ban), and NEI CORE grant EY-00785 to the Department of Ophthalmology and Visual Science, Yale University.

Current address of Y. Ban: Kyoto Prefectural University of Medicine, 465 Kaji-cho Kamigyo-ku, Kyoto, Japan, 602-0841.

REFERENCES


