Apical and Basal Regulation of the Permeability of the Retinal Pigment Epithelium

Shaomin Peng, Christoph Rabner, and Lawrence J. Rizzolo

PURPOSE. The functional characteristics of tight junctions in the outer blood–retinal barrier change during embryonic development and in the presence of disease. A culture model of developing retinal pigment epithelium (RPE) was used to examine the regulation of the tight junctions.

METHODS. RPE from chick embryos was cultured on filters that separated the apical and basal medium compartments. Cultures were maintained in various combinations of serum-free medium, serum-free medium that was conditioned by neural retinas, or serum-free medium that was supplemented with bovine pituitary extract, serum, or various hormones. Function was monitored by the transepithelial electrical resistance (TER) or the permeation of small organic tracers. Structure was monitored by immunofluorescence and freeze-fracture electron microscopy.

RESULTS. Functional analysis indicated differences in permeability among RPE of different embryonic age and culture conditions. In serum-free medium, the tight junctions were leaky or failed to form. Barrier properties increased if pituitary extract was added to the basal medium chamber or retina-conditioned medium was added to the apical chamber. Retina-conditioned medium was more effective at organizing tight junctional strands into a continuous network, but bovine pituitary extract appeared to modulate the permeability of that network. In combination, they synergistically elevated the TER to physiological levels. Although the thyroid hormone T3 had no effect, serum in the apical medium chamber inhibited the ability of RPE cells to respond to retina-conditioned medium.

CONCLUSIONS. Diffusible factors secreted by the neural retina acted synergistically with basolateral stimulation to regulate the structure and function of RPE tight junctions. Serum on the apical side of the RPE monolayer inhibited the ability of retinal factors to upregulate the tight junction barrier. (Invest Ophthalmol Vis Sci. 2003;44:808–817) DOI:10.1167/iovs.02-0473

The retinal pigment epithelium (RPE) lies at the interface between the neural retina and the choroid. The microvilli of its apical surface interdigitate with the outer segments of photoreceptors, whereas its highly infolded basal membrane interacts with Bruch’s membrane, serum components that cross the fenestrated choriocapillaris, and the secretions of the various choroidal cell types. The effect of the RPE on the properties of the neighboring cells is well documented, but the effects of neighboring environments on the RPE are less well studied. During pathogenesis, these environments can change. The accumulation of drusen in Bruch’s membrane may alter basal interactions, whereas a break in the RPE monolayer would allow serum to reach the apical surface of the epithelium. Culture studies suggest that subretinal serum further degrades the RPE barrier. A defining characteristic of simple, transporting epithelia is the circumferential band of tight junctions that seal the space between neighboring cells of the monolayer. Tight junctions are semiselective in their ability to retard the transepithelial diffusion of solutes across these paracellular spaces. Furthermore, the permeability and selectivity of the junctions can be regulated physiologically and pharmacologically. During embryogenesis, the tight junctions of RPE cells become progressively less permeable, and there are changes in selectivity. The process is regulated by the developing neural retina and can be divided into early, intermediate, and late stages. We devised a primary culture of chick RPE cells that models the transition from the early to the intermediate stage. RPE cells were isolated from the early (embryonic day [E] 7) or intermediate (E14) stage and cultured on filters in the presence or absence of medium that was conditioned by E14 neural retinas. The tight junctions of this culture model exhibit a variety of functional states of that depend on the embryonic age of the RPE cells and at least two secretory products of the neural retina. One secretory product, required only by E7 RPE cells, is less than 10 kDa; the other is a 49-kDa protein that we named RCM49.

The different functional states of the culture model are defined by three assays that measure different properties of the tight junction. The transepithelial electrical resistance (TER) assay is a classic method in which the resistance to an electrical current across the monolayer is measured. If the TER is less than 1000 Ω/cm², then most of the current passes through the paracellular spaces, and the TER measures the tightness of the tight junctions. A second classic assay is measurement of the permeation of nonionic tracers, such as mannitol, across the monolayer. The TER can vary semi-independently when RPE cells of different ages are cultured with or without retina-conditioned medium, which suggests that different functional states of the tight junctions exist.

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In this report, we describe an improved culture medium for producing retina-conditioned medium. We removed bovine pituitary extract from the previous formulation to make a chemically defined medium. This simplified medium allowed the examination of various effectors of the RPE and stimulated retinal secretion 10-fold. Remarkably, a synergistic effect on permeability was observed when retina-conditioned medium was placed in the apical medium chamber and bovine pituitary extract was included in the basal chamber. This synergy was inhibited by serum in the apical medium chamber.

**Materials and Methods**

**RPE Cell Culture**

RPE was isolated from E7 and E14 White Leghorn chicken embryos as described previously. Unless noted, reagents were obtained from Sigma (St. Louis, MO). Single cell suspensions were plated on laminin-coated (Upstate Biotechnologies, Lake Placid, NY) filters (Transwell; Costar, Cambridge, MA) at a density of 5 \times 10^4 cells/cm^2. Trypsinization, washing, and repopulation of the laminin-coated filters were performed according to the supplier's instructions, with 7.5 (E7) or 10 (E14) \mu g laminin per filter, and used the same day. The plating medium varied according to the experimental protocol. Cells were plated in SF3 medium or modifications of SF3 (SF2, SF3+, and SF4). SF3 is a completely defined medium that contains Dulbecco's modified Eagle's medium (Invitrogen Corp., Grand Island, NY) supplemented with ITS+ (6.3 \mu g/mL insulin, 6.3 \mu g/mL transferrin, 6.3 \mu g/mL selenium acid, 1.25 mg/mL BSA, and 5.4 \mu g/mL linoleic acid; BD Biosciences, Bedford, MA, 20 ng/mL hydrocortisone, 8 ng/mL progesterone, 292 \mu g/mL l-glutamine, 110 \mu g/mL pyruvic acid, and antibiotics. SF3+ is SF3 supplemented with 0.25% fetal bovine serum. SF2 medium is SF3 supplemented with 50 \mu g/mL bovine pituitary extract (Upstate Biotechnologies). SF4 medium is SF3 supplemented with 5 \mu g/mL linoleic acid; BD Biosciences, Bedford, MA), 20 ng/mL hydrocortisone, 8 ng/mL progesterone, 292 \mu g/mL l-glutamine, 110 \mu g/mL pyruvic acid, and antibiotics. SF3+ is SF3 supplemented with 0.25% fetal bovine serum. SF2 medium is SF3 supplemented with 5 \mu g/mL bovine pituitary extract (Upstate Biotechnologies). SF4 medium is SF3 supplemented with 10 \mu g/mL 3,5',5-triiodo-l-thyronine (T3), 20 \mu g/mL epidermal growth factor, and 5 \mu g/mL basic fibroblast growth factor (bFGF; Invitrogen Corp.).

After 1 day in culture, the media in the apical and basolateral chambers were changed according to the experimental protocol. In some experiments, the medium in the apical chamber was replaced by retina-conditioned rcsF3, which is SF3 conditioned by E14 neural retinas, by a published method. Depending on the experimental protocol, these media were supplemented with various concentrations of fetal calf serum or T3, as described in the text.

**Assays of Functional Permeability**

All assays of permeability were performed after cells were 9 days in culture, and tests of significance were performed with the Students t-test. TER was measured using endohm electrodes (World Precision Instruments, Sarasota, FL). Measurements were made in a modified SF3 in which the bicarbonate of DMEM was replaced with 20 mM HEPES (pH 7.2). The background resistance (10 \Omega) was subtracted and the measurement reported as ohms times square centimeter.

**Immunofluorescence**

After 9 days in culture, cells were fixed with 2% paraformaldehyde in PBS for 20 minutes at room temperature, washed twice in PBS, and quenched with 50 mM ammonium chloride in PBS for 20 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature and blocked with PBS containing 1% BSA, 5% goat serum, and 0.05% sodium azide for 1 hour at room temperature or overnight at 4°C. To label ZO-1, cultures were incubated for 1 hour at room temperature in blocking buffer containing 1:200 rabbit-anti-ZO-1 polyclonal antibody (Zymed Laboratories Inc., San Francisco, CA). After washing with PBS, the cultures were incubated with 1:500 FITC-conjugated goat-anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) in PBS containing 1% BSA and 5% goat serum for 45 minutes at room temperature. To label actin filaments, the cultures were fixed as above and labeled with phalloidin (Bodipy 581/591; Molecular Probes, Inc., according to the manufacturer's instructions. The cells were viewed with an immunofluorescence microscope (Axioskop; Carl Zeiss, Inc., Thornwood, NY). Images were captured (Spot camera; Diagnostic Instruments, Sterling Heights, MI) and prepared with image-analysis software (Photoshop; Adobe Systems, Inc., San Jose, CA). Because of its effect on permeability, cell density was determined by counting cells in five randomly selected fields (95,000–94,000 \mu m^2). Cell counts were performed using NIH Image software (ver. 1.60; by Wayne Rasband, National Institutes of Health, Bethesda, MD; available by ftp at zippy.nimh.nih.gov or at http://rsb.info.nih.gov/nih-image). Statistical significance was determined using the Student's t-test.

**Immunoblots**

A monoclonal antibody to actin (JLA20) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Polyclonal antibodies to occludin had been raised against the C-terminal cytoplasmic domain and characterized in chick RPE. Protein extracts were prepared, resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted as described. The level of actin staining was used as an internal standard to normalize the data.

**Freeze-Fracture**

After cells were 9 days in culture, the TER was measured and three to five filters for each culture condition were fixed with 2.5% glutaraldehyde (Polyscience, Inc., Warrington, PA) in 0.1 M cacodylate buffer (pH 7.2) for 20 minutes at RT. Filters were rinsed three times for 30 minutes each in 0.1 M cacodylate buffer and processed for freeze-fracturing as described. For each experimental condition, five specimens and replicas were prepared from each filter. Electron micrographs of the planar complex were acquired on an electron microscope (model 1010; Philips, Mahwah, NJ) and at a primary magnification of 38,000\times. Electron micrographs were digitized at 1200 dpi with a scanner (Powerlook 1100; Umax Technologies, Fremont, CA) and processed on computer (Photoshop; Adobe). For constant orientation of the tight junctions, images were rotated to the appropriate angle.

**Results**

**Sensitivity of E7 and E14 RPE to Culture Conditions**

To examine the requirement for bovine pituitary extract in our culture model, we deleted it from our previous medium formulation (SF2) to make SF3 medium. RPE cells were isolated from E7 or E14 embryos and cultured in SF3 alone. Neither E7 nor E14 RPE cells adhered tightly to the laminin-coated filters. Many cells were removed with the gentlest washing. Consequently, the cultures exhibited a very low TER (Table 1). For comparison, a layer of fibroblasts yielded a TER of approximately 10 \Omega \times cm^2. The ability of E7 RPE cells to adhere to the filters was improved by adding 0.25% serum during the initial plating step. Serum was discontinued after day 1 in culture, because its continued presence inhibited the response to retina-conditioned medium (see Fig. 8). Confluent monolayers were obtained with a TER of 23.9 \Omega \times cm^2. This was
slightly lower than previously reported for E7 RPE cells cultured in SF2, but phalloidin staining demonstrated that the cultures were confluent with circumferential bands of actin that gave the cobblestone appearance typical of epithelial monolayers (Fig. 1). The absence of fusiform cells or cells with stress fibers indicated there was little or no contamination by fibroblasts. The tight junction protein ZO-1 was also distributed in circumferential bands where neighboring cells met. The culture improved slightly when SF2 replaced SF3 in the basal medium chamber. Under these conditions, a trend toward higher TER (25.7 ± 0.7 Ω × cm$^2$) was observed, but the increase failed to reach statistical significance ($P < 0.07$). This was similar to the TER previously reported when E7 RPE cells were cultured with SF2 in the apical and basal media chambers.

By contrast, it was difficult to obtain confluent monolayers of E14 RPE cells when they were cultured in SF3, even if serum was included in the plating medium or if the filters were preincubated in serum. Nonetheless, if the cells were not washed and the medium gently removed and replaced during feeding, a confluent culture could be obtained. However, the TER was only $17.5 ± 0.8$ Ω × cm$^2$. Actin staining revealed large, irregularly shaped cells that contained stress fibers. The presence of melanin granules indicated these were RPE cells and not fibroblasts. SF3 is similar to the defined medium used by Chang et al. for the culture of rat RPE, but has no T3, epidermal growth factor (EGF), or fibroblast growth factor (FGF). Inclusion of these hormones in various concentrations (SF4 media) did not improve the plating efficiency, raise the TER (data not shown), or restore the distribution of actin and ZO-1 (Fig. 2). Small gaps in the monolayer were often evident. Although some ZO-1 was distributed along cell-cell contacts, it was mostly observed in the cytoplasm and nucleus. ZO-1 rarely aligned along stress fibers.

### Table 1. Effect of Culture Conditions on the TER of E7 and E14 RPE Cells

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7</td>
<td>12.6 ± 1.4</td>
<td>25.9 ± 0.6</td>
<td>25.7 ± 0.7</td>
<td>52.9 ± 3.3</td>
<td>77.3 ± 1.8</td>
<td>80.2 ± 5.2</td>
</tr>
<tr>
<td>E14</td>
<td>17.5 ± 0.8</td>
<td>16.2 ± 2.6</td>
<td>35.5 ± 0.6</td>
<td>74.7 ± 5.3</td>
<td>88.5 ± 2.7</td>
<td>143.1 ± 9.1</td>
</tr>
</tbody>
</table>

Data were analyzed from two to nine experiments using three to six times square centimeter ± SE are indicated.

* Serum (0.25%) was present only in the initial plating step.
† SF2 was used in the initial plating step and thereafter in the basolateral chamber. The TER in ohms times square centimeter ± SE are indicated.

### Figure 1. In E7 RPE cells, serum or bovine pituitary extract promoted the formation of an epithelioid monolayer. RPE was isolated from E7 embryos and cultured with SF3 medium (no pituitary extract) using two protocols. (A, B) Serum (0.25%) was added to SF3 in the plating step to promote adhesion, but was excluded for the remainder of the culture period. (C, D) SF2 medium (SF3 supplemented with pituitary extract) was used to plate the cells and thereafter was added to the basolateral medium chamber. After plating, SF2 was replaced with SF3 in the apical medium chamber. After 9 days, some cultures were fixed and stained with phalloidin to reveal the distribution of actin fibers (A, C). Other cultures were stained to reveal the distribution of ZO-1 by using a protocol for immunofluorescence. Each procedure revealed a continuous monolayer with circumferential bands of actin and ZO-1.

### Synergistic Action of Retinal Secretions and Bovine Pituitary Extract

Retina-conditioned medium induced an epithelioid phenotype, even without bovine pituitary extract. E14 neural retinas were used to condition SF3 medium. The rcSF3 medium stimulated an increase in the TER of both the E7 and E14 cultures (Table 1, columns 4 and 5). The effect was especially dramatic in E14 cultures, where rcSF3 restored an epithelioid phenotype to the cells (Fig. 3). Circumferential bands of actin and a cobblestone appearance were observed when actin filaments were fluorescently labeled with phalloidin. ZO-1 was redistributed to the lateral membranes. There was an absence of stress fibers or irregularly shaped cells.

Both retinal factors and bovine pituitary extract promoted cell adhesion and the formation of lateral contacts, as demonstrated by the inability to simply rinse away cells and by the
redistribution of actin and ZO-1. In principal, the promotion of adhesion along the basolateral membranes of an epithelial cell may be sufficient to induce the formation of tight junctions. Notably, the combination of SF2 and rcSF3 was more than additive. This was determined by plating E7 or E14 RPE in SF2 and culturing for 1 day. Then, the medium in the apical medium chamber was replaced with SF3 or rcSF3 and cultured for eight more days. In each case, the cells displayed an epithelial phenotype and a transepithelial electrical resistance (Figs. 1, 2, 4; Table 1 columns 3 and 6). The contributions of SF2 alone or rcSF3 alone can be estimated from Table 1 by subtracting column 1 from column 3 or column 4, respectively. By adding the sum of these contributions back to column 1, the predicted TER for column 6 should be 66 \( \Omega \times \text{cm}^2 \) for E7 and 92.7 \( \Omega \times \text{cm}^2 \) for E14 cultures. Instead, 80.2 and 143.1 \( \Omega \times \text{cm}^2 \), respectively, were observed.

Compared with SF2 in earlier studies, SF3 medium was more effective in promoting the secretion of retinal factors that increase the electrical resistance of tight junctions. In each of nine independent experiments, the TER was higher than that previously reported for RPE cultured with retina-conditioned SF2 (rcSF2).\(^{10}\) For E7 RPE, the TER (Table 1, column 6) was more than double the TER of RPE maintained in rcSF2. In E14 RPE, the TER was higher than the 80 to 100 \( \Omega \times \text{cm}^2 \) obtained in rcSF2. Occasionally, a TER of 200 \( \Omega \times \text{cm}^2 \) was obtained (see Fig. 7). Viewed another way, published data demonstrate that rcSF2 increases the TER up to twofold in E7 and E14 cultures. By contrast, rcSF3 increased the TER more than threefold (Table 1; compare columns 3 and 6). When retina-conditioned media were prepared in the presence of \(^{35}\)S-methionine, 10 times more label was incorporated into secretory products that were precipitable by trichloroacetic acid. When

![Figure 2](image1.png)  
**Figure 2.** In E14 RPE, bovine pituitary extract, but not serum, promoted the formation of an epithelioid monolayer. RPE was isolated from E14 embryos and examined using the protocols described in the legend to Figure 1. In addition, cells were cultured with SF4 medium (SF3 supplemented with T3, FGF, and EGF). (A, B) Serum included in the plating step. (C, D) Cells were cultured in SF3 (apical chamber) and SF2 (basolateral chamber). (E, F) Cells were cultured in SF4. (A, C, E) Actin fibers revealed by phalloidin. (B, D, F) ZO-1 revealed by immunofluorescence. In cultures without SF2 in the basolateral chamber, the cells were large, contained stress fibers (arrows), and showed no circumferential bands of actin or ZO-1. Clumps of melanin granules (arrowbeads) verified that these were RPE cells. Contents of the two media chambers are indicated as apical over basolateral (left). Bar, 10 \( \mu \text{m} \).

![Figure 3](image2.png)  
**Figure 3.** Retina-conditioned medium induced the epithelioid phenotype. RPE cells were isolated from E7 (A, B) or E14 (C, D) embryos and cultured with SF3 in the basolateral chamber and rcSF3 in the apical chamber. The distributions of actin (A, C) and ZO-1 (B, D) were revealed as described in the legend to Figure 1. Although the E7 cultures appeared similar to those depicted in Figures 1A and 1B, retina-conditioned medium had a profound effect on the E14 cultures. The monolayers displayed a typical cobblestone appearance with circumferential bands of actin and ZO-1 and without stress fibers. Bar, 10 \( \mu \text{m} \).
resolved by gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the secretory products appeared to be the same as those that have been reported for rcSF2 (Ref. 14 and data not shown). To determine whether the presence of pituitary extract in the apical medium chamber inhibits the retinal factors, extract, in the amount found in rcSF2, was added together with rcSF3 to the apical medium chamber. No statistical difference in the TER was observed between rcSF3 and rcSF3 + pituitary extract (P < 0.4).

Although both bovine pituitary extract (SF2) and retina-conditioned SF3 medium were able to induce the epithelial phenotype, the combination of the two had a synergistic effect on the TER. Accordingly, SF2 was present in the basolateral chamber of the following experiments. All changes in media composition refer exclusively to the apical medium chamber.

Effect on the Permeation of Mannitol and N-Acetyleneuraminic Acid

The TER measures one aspect of junctional permeability. The inverse of the TER is proportional to the conductance of ions down a voltage gradient. By contrast, tracer studies measure passive diffusion. Using the small organic tracer, mannitol, studies have shown that different mechanisms regulate TER and the permeation of mannitol.16–18 Further, RPE differentially regulates the permeation of mannitol and a similarly sized anionic sugar, N-acetyleneuraminic acid.19 Because the new culture conditions described herein yielded cultures with increased TER, we asked how the new conditions would affect the permeation of mannitol and the permeation ratio of N-acetyleneuraminic acid/mannitol (Table 2).

The permeation of mannitol was lower in E14 cultures than in E7 cultures, and the magnitude of the difference was similar to the difference in ion conductance. In SF3, the permeation of mannitol was 27% lower in E14 RPE, compared with a 28% lower conductance. In rcSF3, the permeation of mannitol was 41% lower in E14 RPE, compared with a 44% lower conductance. The N-acetyleneuraminic acid/mannitol ratio was slightly, but significantly (P < 0.001), lower in E14 cultures when RPE was maintained in SF3. This ratio was decreased by rcSF3, but in this case the difference between the E7 and E14 cultures was not significant (P < 0.15).

In the E7 cultures, rcSF3 decreased the permeation of mannitol 73%. The effect on ion conductance was nearly the same, 68%. The N-acetyleneuraminic acid/mannitol ratio decreased 20%, which indicates that the permeation of N-acetyleneuraminic acid decreased more than mannitol. Similarly, in the E14 cultures, rcSF3 decreased the permeation of mannitol 77% and the conductance 75%. The rcSF3 medium also decreased the N-acetyleneuraminic acid/mannitol ratio, but as noted, the magnitude of the effect was lower than for the E7 cultures.

Cell Density

A decrease in cell density decreases the length of the tight junctions per square centimeter of monolayer. Because much more electrical current is carried across the paracellular than the transcellular path, a decrease in cell density would increase the TER.19 E7 RPE is more dense than E14, both in vivo and in vitro, but cell density is not affected by rcSF2.13,23 To determine whether SF3 affects cell density, we measured the density of the cells from micrographs similar to those in Figure 4. The data reported in Table 3 are close to that reported for RPE maintained in SF2 or rcSF2.13 The retina-derived factors had no effect on cell size. As in vivo, E7 cells were slightly, but significantly (P < 0.005), smaller than E14 cells. The 30% lower cell density theoretically would increase the TER 15%.20 This physical difference was unable to account for the entire difference in TER (Table 1) or the permeation of mannitol (Table 2) between E7 and E14 cells. The change in cell size cannot account for the differences in another property of the tight

Table 2. Permeation of Mannitol and N-Acetyleneuraminic Acid

<table>
<thead>
<tr>
<th>SF3 Medium</th>
<th>rcSF3 Medium</th>
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<tbody>
<tr>
<td>Mannitol†</td>
<td>NA/Mannitol†</td>
</tr>
<tr>
<td>E7</td>
<td>1.04 ± 0.02</td>
</tr>
<tr>
<td>E14</td>
<td>0.76 ± 0.04</td>
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</table>

Data are the mean ± SE of results in 6 experiments.
† Permeability in microliters per square centimeter per minute.

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Table 3. Cell Density as a Function of Age and Culture Conditions

<table>
<thead>
<tr>
<th>Age</th>
<th>SF3</th>
<th>rcSF3</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7</td>
<td>7.7 ± 0.7</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td>E14</td>
<td>5.5 ± 0.1</td>
<td>5.4 ± 0.1</td>
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</table>

Cells were cultured and stained as described in the legend to Figure 4. Cells were counted in three randomly selected fields, each approximately 100,000 μm². Data are mean cells per square centimeter × 10⁻⁴ ± SE.

Occludin Expression

Although the level of occludin expression does not change during embryonic development, there is an increase in the higher-molecular-weight forms that suggests increased phosphorylation. The expression of occludin was monitored by immunoblot analysis (Fig. 5). There was little difference in the level of expression between E7 and E14 cultures or as a function of rcSF3. Nor was there any change in the distribution among high- and low-molecular-weight forms of the protein, which suggests no overt changes in phosphorylation. In the E7 and E14 cultures, more occludin was found in the higher-molecular-weight forms than previously reported for freshly isolated RPE. Such discrepancies between the in vivo and in vitro expression of occludin and other proteins is common. These data indicate that rcSF3 did not regulate the expression or posttranslational modification of occludin, and that phosphorylation of occludin was insufficient to affect permeability in these conditions.

Freeze-Fracture

There is evidence for some correlation between permeability and the structure of the tight junctions. Therefore, we examined the structure of the tight junctions by freeze-fracture electron microscopy. When E7 RPE was cultured with SF3 in the apical and basal media chambers, junctional strands were observed with intermixed gap junctions (Fig. 6A). Many of the strands were interconnected to form a continuous tight junction. In this network, some strands were connected at only one end, and free-floating strands were also observed. By contrast, junctional strands were difficult to find when E14 RPE was cultured in SF3 alone. Strands that were observed were usually free-floating or organized in small patches of interconnected strands (Fig. 6B). No evidence was observed of an extended network of interconnected strands that was capable of forming a diffusion barrier. When E14 cultures were maintained with SF2 in the basal medium chamber, the cells were more adherent, and junctional strands were much easier to find (Fig. 6C). Regions of junctional network (lower left of the image) alternated with regions of unconnected strands (upper right of the image). The rcSF3 medium was more effective than SF2 in forming a continuous junctional network (Fig. 6E). This effect was also reflected in a higher TER (Table 1, columns 3 and 4). The combination of rcSF3 in the apical chamber and SF2 in the basal medium chamber also resulted in a well-organized junctional complex (Fig. 6F). Even though this combination synergistically elevated the TER (Table 1, column 6), the depth of the tight junctions (perpendicular distance from apical-most to basal-most strand) appeared to be less than in the cultures with rcSF3 alone. There was no obvious difference in the number or complexity of the strands. These data indicate that retinal secretions and pituitary extract affected the fine structure of tight junctions in different ways.

Effect of T3 on the TER of the RPE

The thyroid hormone T3 is thought to promote epithelial properties of the RPE and other epithelia. It is also a factor in the development and maintenance of the neural retina. Therefore, we tested whether T3 affects the barrier properties of the RPE directly or indirectly, by altering the secretions of the neural retina. No differences were observed between SF3 and SF3 containing T3, either in plating efficiency or properties of the cultured RPE (data not shown). Accordingly, experiments were performed with SF2 in the basal medium chamber and SF3 containing various amounts of T3 in the apical chamber. To test the hypothesis that T3 affects the RPE indirectly by altering the secretions of the neural retina, SF3 containing various amounts of T3 was used to produce retina-conditioned media, which was then added to the apical medium chamber. No effect was observed in either experiment when T3 was added in the range of 1.0 to 1000 pM (Fig. 7).

Effect of Serum in the Apical Chamber on the Effects of Retina-conditioned Medium

Serum increases the permeability of rodent RPE when added to the apical medium chamber. Two experimental paradigms were used to investigate the effects of apical serum in chick RPE. E7 and E14 RPE were maintained with SF2 medium in the basolateral chamber, and serum was added to rcSF3 medium in the apical medium chamber. Alternatively, to test the effects of serum on the secretions of the retina, rcSF3 was prepared in SF3 that contained serum. For each age, serum decreased the TER without overt effects on cell morphology (morphology data not shown). Only 1.7% serum was needed to lower the TER of E7 cultures 25% and E14 cultures 40% (Fig. 8). Higher levels of serum slightly decreased the TER further. In 6.7% serum, the TER for the E7 and E14 cultures was approximately the same but was still greater than the TER for E14 cultured with SF3 in the apical medium chamber (Table 1). Serum had little or no effect on the TER of RPE cultured in non-retina-conditioned medium, regardless of whether it was added to the apical or basolateral medium chambers (data not shown). There was little or no difference if the rcSF3 was prepared in

Figure 5. The expression of occludin was unaffected by retina-conditioned medium. After 9 days, the indicated cultures were extracted with sodium dodecyl sulfate. The extracts were resolved by gel electrophoresis and immunoblot analysis was conducted with antibodies to occludin or actin. As a control, parallel samples were analyzed by immunoblot with nonimmune rabbit antiserum. Neither the level of expression nor the distribution among isoforms was affected by retina-conditioned medium or the embryonic age of the cells at the time of isolation.
the presence of serum or if serum was added after the medium was prepared, which suggests that serum had little or no effect on the ability of the retina to secrete factors that increase the TER.

**DISCUSSION**

It is well established that cell interactions at the basal and lateral plasma membranes induce the epithelial cell phenotype. Nonetheless, studies have demonstrated that apical interactions of the RPE with the neural retina modulate the cellular polarity that is induced by basolateral interactions. The present study extends this concept by demonstrating that basal and apical environments acted synergistically to regulate RPE tight junctions. RPE could be maintained in SF3, but only E14 RPE formed confluent monolayers with functional, albeit leaky, tight junctions. This finding indicates that the E14 RPE needed more environmental cues than E7 RPE to reestablish an epithelioid phenotype in culture and is consistent with earlier observations that E14 RPE is more sensitive to culture conditions. Two medium supplements enabled E14 RPE to adopt an epithelial phenotype. Bovine pituitary extract (SF2 medium) contains a collection of hormones and growth factors that would normally be presented through the blood stream to the basal side of the RPE. The extract promoted the adhesive-ness of the monolayers, as noted by their increased stability during washing and refeeding, along with promoting an epithelial morphology. The surprising finding was that factors secreted by the neural retina (rcSF3 medium) induced the epithelial phenotype. Furthermore, retinal secretions were more effective than pituitary extract at establishing a continuous network of tight junctions. This was an unusual demonstration that interactions mediated by the apical plasma membrane could induce an epithelial phenotype. It is equally remarkable that the major effect on permeability of basally applied pituitary extract was to enhance the efficacy of the retinal secretions.

Retina-conditioned medium acted principally by organizing tight junctional strands into an anastomosing network that completely encircled the cell. In E7 RPE, this meant incorporating free-floating strands into an existing network and attaching the free end of those strands that were attached to the network by only one end. By contrast, in SF3 the junctional strands of E14 RPE were rare and completely disorganized. Although pituitary extract induced circumferential bands of actin and ZO-1, the freeze-fracture data indicated rcSF3 was more effective in forming a network of strands that was free of discontinuities. Compared with cultures maintained with SF2 in the basal medium chamber, the combination of apical rcSF3 with basolateral SF2 increased the TER three to four times and decreased the permeation of mannitol by approximately 75%. By contrast, the combined media decreased the permeation of N-acetylneuraminic acid more than that of mannitol. This confirmed our earlier report that individual strands of the tight junction discriminate between similar sized organic molecules that differ in charge. By closing discontinuities in the network and increasing the number of strands to be traversed, rcSF3 decreased the N-acetylneuraminic acid/mannitol permeation ratio. Therefore, the effect measured by each assay could be explained by the closing of discontinuities in the junctions. There is no need to postulate effects on the permeability of individual strands. By contrast, pituitary extract appeared to
that phosphorylation changes. However, in the present study, neither the level of expression nor the distribution of occludin among its various isoforms was altered by age or culture condition. Because occludin appeared to be more phosphorylated in culture than in vivo, these data suggest that phosphorylation alone was insufficient to induce high TER or low permeability of mannitol.

By contrast, retina-conditioned medium regulates the expression of the transmembrane protein, claudin 5. Claudins readily form junctional strands even in the absence of the other transmembrane proteins. Claudin 5 is transiently expressed by embryonic RPE with a peak expression on E10. In cultures of E7 RPE, the expression of claudin 5 is induced by rcSF2, but in E14 RPE the expression of claudin 5 is reduced. It is unknown which claudin(s) replace claudin 5 after E10. The extracellular domains of the twenty known claudins show charge differences that could account for the variation in permeability and selectivity among tight junctions in different epithelia and endothelia. Therefore, a difference in the subset of claudins that are expressed on E7 and E14 could account for the differences in permeability.

**Effects of Apical T3 and Serum**

The use of a defined medium allowed us to test candidate hormones to determine whether they affect tight junctions through direct effects on the RPE or indirectly by inducing the retina to secrete active factors. The thyroid hormone T3 was investigated, because different T3 receptors are expressed by various cells in the neural retina during development. They are especially prominent in the photoreceptor precursors on E14. Further, T3 receptors are expressed by human fetal RPE, and serum-free media devised for rat RPE contain T3. The only binding protein for T3 that was present in SF3 was albumin. Because of albumin’s low affinity (Kd = 1 × 10^-5 M) and competition by linoleic acid, the concentrations of T3 that were used (1-1000 pM) probably span the physiologic

![Figure 7](image7.png)

**Figure 7.** T3 did not affect the TER of cultured RPE directly or indirectly through effects on the retina. E7 (squares) or E14 (circles) RPE cells were cultured with SF2 in the basolateral medium chamber. The apical chamber contained SF2 (closed symbols) that was supplemented with the indicated concentration of T3, or contained rcSF3 (closed symbols) that was conditioned in the presence of the indicated concentration of T3. The intercept at the y-axis indicates the TER of control cultures without T3. Error bars are the standard deviation of results in three filters and are often smaller than the symbol. Similar results were obtained in repeated experiments.

![Figure 8](image8.png)

**Figure 8.** Serum in the apical medium chamber inhibited the ability of retina-conditioned medium to increase the TER. E7 (squares) or E14 (circles) RPE was cultured with SF2 in the basolateral chamber. The indicated amounts of serum were added to rcSF3 (open symbols) or to SF3 that was used to make rcSF3 (closed symbols). Error bars are the standard deviation of results in three filters and are often smaller than the symbols.
range of free T3. T3 failed to affect the TER, either by directly affecting the RPE, or indirectly through the production of active factors by the neural retina.

Studies have indicated that serum applied to the apical surface increases the permeability of the rodent RPE. Although we observed no effect on the TER when SF3 was in the apical medium chamber, even low levels of apically applied serum inhibited the effects of cSF3. The effect of serum was specific, because it did not inhibit secretion by the neural retina or the effects of pituitary extract on the RPE cultures. Of note, a high concentration of bovine serum albumin was present in all media. Therefore, the inhibition was not due to nonspecific effects of high protein concentration in the apical medium chamber. The difference between our results and those in earlier studies lies in the ability of the other investigators to obtain a high TER with a cocktail of growth factors instead of retina-conditioned medium. We tried several variations of this medium (data are shown for SF4), but did not find a formulation that was compatible with chick RPE. Nonetheless, in our study and those of Chang et al., the factors that induced a high TER were inhibited by apical serum.

The TER of chick RPE in situ is 138 Ω × cm². By eliminating factors that are not normally found in the subretinal space, we produced a more active retina-conditioned medium that induces barrier properties in the physiological range for chick RPE. This model system should prove useful for identifying factors that enhance or degrade barrier properties of the RPE, and examining the signaling pathways that are activated. There are important implications for transplantation procedures that introduce healthy RPE into a diseased environment that no longer provides the necessary apical and basal interactions.

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References