Localization of cellular retinol-binding protein in bovine retina and retinal pigment epithelium, with a consideration of the pigment epithelium isolation technique

John C. Saari, Ann H. Bunt, Sidney Futterman, and Elaine R. Berman

Bovine RPE was isolated by commonly used brushout procedures and analyzed by light and electron microscopy. The preparation was found to consist almost entirely of cells with retained organelles (mitochondria, pigment, and other granules) but with broken surface membranes and extracted cytoplasm. In keeping with this, the wash obtained by sedimenting these broken cells contained approximately 97 percent of the cellular retinol-binding protein present in the suspension. Cellular retinoic acid-binding protein, present in bovine retinal extracts, was found in low amounts in the wash from RPE. The cellular retinol-binding protein present in the RPE wash was of high specific activity and similar in properties to that obtained from bovine retina. Supernatant obtained from sonicated rod outer segments contained approximately 10 percent of the retinol-binding protein of the retina. No retinoic acid-binding protein was found. The relatively large amount of cellular retinol-binding protein present in the RPE (more than is found in the retina) is consistent with a functional role of this protein in uptake and transport of retinol by the RPE.

Key words: retinal pigment epithelium, retinol, cellular retinol-binding protein, cellular retinoic acid-binding protein.

The retinal pigment epithelium (RPE), situated between the choroidal vasculature and the neural retina, serves as a barrier to the free diffusion of large molecules from the bloodstream to the photoreceptor cells. Retinol, bound initially to the serum retinol-binding protein within the choroid, must traverse this single layer of epithelial cells to gain access to the photoreceptors, the primary site of vitamin A function in the visual system. Uptake of retinol from the serum may be dependent upon recep-
Figs. 1 to 7. Figs. 1 to 5 are light photomicrographs of 1 μm Epon sections stained with Richardson's methylene blue-azure II mixture; Figs. 6 and 7 are electron micrographs stained with uranyl acetate and lead citrate. For individual legends see opposite page.
tors located on the basal surface of the RPE, which recognize the serum retinol-binding protein-retinol complex. Several laboratories have recently reported the existence of intracellular retinol-binding proteins in eye, retina, and pigment epithelium. These proteins may serve as acceptors for serum retinol. The protein nature of the binding fraction has been established by our unpublished observations that binding of retinol is abolished by pronase digestion.

Initial attempts to study the internalization and subsequent fate of serum retinol were focused on the detection, quantification, and characterization of the cellular retinol-binding protein in isolated bovine RPE cells. During the course of these investigations, unanticipated difficulties were encountered in the preparation of suspensions of intact RPE cells. This article describes these findings, documenting the presence of cellular retinol-binding protein in RPE and comparing the properties of the protein from this tissue with those already described for the protein from retina.

Materials and methods

All-trans-retinal, 9-cis-retinal, all-trans-retinol, all-trans-retinoic acid, and retinyl acetate and palmitate were purchased from Eastman Organic Chemicals (Rochester, N. Y.). [1-3H]Retinol (2.66 Ci./mmol.) was purchased from New England Nuclear Corp. (Boston, Mass.). [11-12-3H]Retinoic acid 1.45 Ci./mmol.) was generously provided by Dr. W. E. Scott of Hoffmann-La Roche, Inc. (Huntley, N. J.). Cattle eyes were obtained from a local slaughterhouse. Bovine serum albumin was from Pentex, Inc., (Kankakee, Ill.); ovalbumin, aldolase, carbonic anhydrase, myoglobin, hemoglobin, collagenase, and hyaluronidase were from Sigma Chemical Co. (St. Louis, Mo.).

Isolation of bovine pigment epithelial cells. Eyes were dissected under normal room illumination and the anterior part was discarded. The retina was teased out with a small forceps and cut at the optic nerve head. A 1 to 2 ml. amount of cold medium (usually 0.32M sucrose, but see Results section for variations) was added to the eye cup, and pigment epithelial cells were released with a small brush. Monitoring the procedure with a dissecting microscope showed that very light brushing was sufficient to remove the monolayer of cells, whereas more vigorous brushing disrupted Bruch's membrane and released elements of the choroid. The suspension of cells was removed with a fire-polished, wide-orifice Pasteur pipette, and the cells were washed as described by Berman and Feeney.

Morphologic analysis. In each experiment, the posterior portion of an eye was fixed for morphologic analysis after removal of the vitreous, and another after removal of the vitreous and neural retina. The eye cup was fixed in 2 percent glutaraldehyde-0.18M phosphate buffer, pH 7.4, for approximately 16 hr. at 5° C. A portion of the RPE suspension was removed and added to an equal volume of cold 4 percent glutaraldehyde-0.32M sucrose, pH 7.2. Intact tissues and cellular...
isolates were postfixed in 1 percent OsO₄ in 0.18M phosphate, pH 7.4, for 1 hr. at room temperature, dehydrated through an ethanol series, and embedded in Epon. Sections were cut at 1 µm and lightly stained (5 sec. on a 60° hot plate) with Richardson's methylene blue-azure II mixture. Sections of approximately 900 A were stained for electron microscopy with uranyl acetate and lead citrate.

Pigment epithelial cell wash. After removal of the crude RPE cell suspension and centrifugation as recommended by Berman and Feeney13 (150 × g for 10 min.), the low-speed wash was further clarified by centrifugation for 1 hr. at 109,000 × g. The high-speed wash was obtained by dialysis against 50 mM Tris, pH 7.5, 0.2M NaCl, and concentrated to about 10 ml. with a pressure concentration cell and a UM-10 membrane (Amicon Corp., Lexington, Mass.).

Isolation of photoreceptor outer segments. Retinas removed from freshly obtained cattle eyes were homogenized in 0.32M sucrose with a loose-fitting glass-glass homogenizer and the suspension was centrifuged for 10 min. at 650 × g to remove nuclei and cell debris. Centrifugation of the supernatant for 10 min. at 1,200 × g sedimented outer segments which were resuspended in 40 percent (w/v) sucrose and layered under 0.15M Tris buffer, pH 7.5. Centrifugation for 15 min. at 30,000 × g floated the outer segments to the interface. After recovery, the flotation process was repeated once more and the outer segments resuspended in 0.15M Tris, pH 7.5.

Regeneration of visual pigments. Regeneration of isorhodopsin from bleached photoreceptor material (0.9 ml.) was effected by the addition of 0.1 ml. of a solution of substrate prepared by dissolving 1 mg. of 9-cis-retinal in 0.1 ml. of 5 percent Triton X-100 in ethanol and diluting to 1 ml. with water. After incubation in the dark at 37° for 1 hr., the concentration of isorhodopsin was determined as described by Futterman and Rollins.14

Assay for retinol-binding proteins. A 1 ml. quantity of extract was incubated for 30 min. at room temperature with 770 pmol. of [3H]retinol (2 µCi.). Then 100 mg. of sucrose were added, and the sample was layered onto a 1.5 by 140 cm. column of Sephadex G-100 which had been equilibrated in 50 mM Tris, pH 7.5, 0.2M in NaCl. Gel filtration was accomplished at 5° C. with a flow rate of 18 ml./hr. Fractions of 3 ml. were collected and analyzed for radioactivity. For displacement studies, a portion of the extract was incubated with 2 µl of [3H]retinol for 10 min., followed by a 30 min. incubation with a 100-fold molar excess of displacing ligand added in 5 µl. of ethanol. The protein concentration of extracts was determined according to the method of Lowry et al.16 with bovine serum albumin as a standard.

Results

Intact bovine retina showed morphologic characteristics previously described14 (Fig. 1) with an anticipated loss of fine structural preservation after several hours of anoxia. Following enucleation, dissection, and removal of the neural retina, the RPE layer appeared intact by light and electron microscopy, with pronounced doming of the apical surfaces, some of which contained phagocytosed outer segments (Fig. 2). RPE layers fixed immediately after removal of the neural retina were almost always intact. When crude suspensions of the RPE were examined by light microscopy, two types of cells were immediately recognizable on the basis of their staining density with Richardson's mixture (Fig. 3). In eyes brushed immediately after decapitation (5 to 15 min., Fig. 3), many of the cells stained darkly blue throughout the cytoplasm. These cells were subsequently found to possess intact plasma membranes when examined by electron microscopy (Fig. 8 and see below). In preparations brushed 1 hr. or more after decapitation, the majority of cells were found to stain poorly with Richardson's stain (Figs. 4 and 5) and appeared pale and washed out. They contained clear cytoplasmic vacuoles which corresponded to swollen mitochondria observed by electron microscopy (Figs. 5 and 9). Both pigmented and nonpigmented cells were found in the isolated sheets, and both types showed the differential staining characteristics.

Electron microscopy of the cell suspensions confirmed the initial impressions obtained by light microscopy. Cells that appeared pale by light microscopy were found to have broken surface membranes, their amorphous cytoplasmic matrix was no longer visible (Figs. 8 and 9), and their mitochondria were swollen and vacuolated (Fig. 9). The basal and apical membranes were most frequently fragmented, whereas the lateral membranes with junctional com-
plexes could often be traced for considerable distances between cells (Fig. 9). Surprisingly, cells with ruptured plasma membranes usually contained abundant intracellular organelles, including nucleus, swollen mitochondria, smooth- and rough-surfaced endoplasmic reticulum, lysosomes, and melanin granules (Figs. 8 and 9). Intact cells, in contrast, possessed a continuous plasma membrane, densely staining amorphous cytoplasm, and smaller, denser, nonvacuolated mitochondria (Figs. 8 and 9).

Irreversible fragmentation of the RPE plasma membrane was found to occur during the brush-out procedure. Removal of the retina from the back of the eye cup appeared to result in a loss of the RPE apical processes (Figs. 6 and 7). The cells, however, appeared intact, suggesting that...
the membrane surface may have reorganized after removal of the retina.

The proportion of intact cells in RPE crude suspensions was very low (<5 percent by cell count) when cells were brushed out with 0.25M sucrose, 0.32M sucrose,12 Hanks' balanced salt solution buffered at pH 7.5 with 10 mM HEPES,2 or 0.25M sucrose buffered at pH 7.5 with 10 mM phosphate, supplemented with 5 mM ATP, Ca++, and Mg++. Attempts to loosen the pigment epithe-

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Fig. 9. Higher magnification of portions of an intact RPE cell on the left and a pale-staining cell on the right. Note intact plasma membrane surrounding cell on left, in contrast to the broken areas of surface membrane (M) of cell on right. Both cells contain mitochondria (*) which are dense and compact in the intact cell, vs. swollen and vacuolated in the broken cell. Most importantly, contrast the densely stained, amorphous cytoplasm (open arrow) of the intact cell with the pale, extracted appearance of the comparable region (filled arrow) of the ruptured cell on the right. R, rough endoplasmic reticulum. (Uranyl acetate and lead citrate; x25,000.)
Table I. Retinol- and retinoic acid-binding activity* of retinal and pigment epithelial cell extracts

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Retinol</th>
<th>Retinoic acid</th>
<th>Retinol</th>
<th>Retinoic acid</th>
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<tbody>
<tr>
<td>Retinal extract†</td>
<td>6</td>
<td>22</td>
<td>57</td>
<td>282</td>
</tr>
<tr>
<td>RPE wash‡</td>
<td>190</td>
<td>8</td>
<td>115</td>
<td>6</td>
</tr>
<tr>
<td>Photoreceptor outer segment extract†</td>
<td>13</td>
<td>§</td>
<td>4</td>
<td>§</td>
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*Assay procedure described in Materials and Methods. The values shown are the average of at least three determinations.
†Extract obtained after homogenization and centrifugation.
‡Wash obtained by centrifugation without prior homogenization.
§Not detectable.

of these contaminants were largely removed after five to six washes of the pellet with sucrose as described by Berman and Feeney.17

Sonication and centrifugation of the crude RPE suspension yielded supernatant fluid containing retinol-binding protein. However, it was not necessary to sonicate the suspension to release the retinol-binding protein into the supernatant (Fig. 10). After mere centrifugation of the suspension, over 97 percent of the total retinol-binding protein of the suspension was found in the supernatant. This result, surprising at first, was entirely consistent with the morphologic observations that the RPE had broken surface membranes and extracted cytoplasm, although superficially appearing intact with retained organelles. Retinoic acid–binding protein, present in threefold to fivefold excess over retinol-binding protein in extracts derived from retina, was present in small amounts in extracts of RPE (Fig. 10 and Table I).

The retinol-binding protein present in the supernatant obtained by centrifugation of crude RPE suspension was found to be of much higher specific activity (Table I) than that found in retinal extracts and hence was more readily purified. Ion-exchange chromatography on DEAE-cel-
Table II. Displacement of \(^{3}H\)retinol from the retinol-binding protein of pigment epithelium

<table>
<thead>
<tr>
<th>Retinoid*</th>
<th>Displaced (%)</th>
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<tbody>
<tr>
<td>Retinol</td>
<td>92</td>
</tr>
<tr>
<td>Retinal</td>
<td>12</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>15</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>0</td>
</tr>
<tr>
<td>Retinyl acetate</td>
<td>0</td>
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</tbody>
</table>

*Added in a 100-fold molar excess after preincubation of the protein with \(^{3}H\)retinol.

from the protein by a 100-fold molar excess of unlabeled retinol. However, retinyl palmitate and retinyl acetate were unable to displace \(^{3}H\)retinol (Table II), and retinal and retinoic acid were relatively ineffective.

The supernatant obtained after sonication of rod outer segments contained retinol-binding protein but not retinoic acid-binding protein (Table I). We estimate that approximately 10 percent of the retinol-binding protein of the retina was recovered in the outer segment fraction.

Discussion

Glocklin and Potts introduced the technique of gently brushing loose the layer of pigment epithelial cells from dissected cattle eyes. Other workers have utilized this procedure, apparently accepting that suspensions obtained were composed of intact cells, since no evidence has been published dealing with the question of cellular integrity in these brushed-out suspensions. We were unable, however, to prepare suspensions of bovine RPE with uniformly intact cells using this commonly utilized technique. When examined by light microscopy, cells were found to occur in sheets and to maintain a semblance of morphologic integrity. However, when analyzed by electron microscopy, these same cells clearly were not intact. The plasma membrane was fragmented along the apical and basal cell borders, and mitochondria appeared ballooned (Fig. 9). Altering the composition of the medium used during brushing of already hypoxic cells failed to appreciably improve the yield of intact cells. In view of this experience, it is quite likely that other investigators...
have used similar preparations of ruptured cells largely lacking cytosol. This may explain the apparent absence of the low molecular weight retinol-binding protein in a study of bovine RPE by Heller.\(^{13}\) Wiggert et al.\(^{10}\) found a low molecular weight binding protein for retinol in brushed-out preparations of bovine RPE. However, their relative yield of binding activity is uncertain, making direct comparisons difficult.

Our finding that nearly all the retinol-binding protein is present in the first wash obtained from RPE cells is consistent with the morphologic finding that nearly all of these cells are not intact. Contaminants of the crude RPE suspension do not contribute significantly to the pool of retinol-binding protein in the wash. Red blood cells do not possess the retinol-binding protein and are mostly intact, as judged by morphology and the small amount of hemoglobin (<10 percent of that obtained after sonication) present in the wash. Photoreceptor outer segments, although present in relatively large amounts, do not contain enough retinol-binding protein to account for the large amount found in the wash. For example, the wash obtained from the RPE suspension contained more retinol-binding protein than the extract from a whole retina, whereas only 3 percent of the rhodopsin of the retina was found in the crude RPE suspension.

The high specific activity retinol-binding protein of RPE allowed easy purification of the protein. Wiggert and Chader\(^{9}\) have previously noted the high specific activity of the retinol-binding protein present in chick embryo RPE. As shown in Fig. 11, the retinol-binding proteins from bovine retina and RPE co-migrated in the SDS-polyacrylamide gel electrophoresis system of Fairbanks et al.\(^{10}\) indicating that their molecular weights are similar, if not identical. For comparison, a sample of bovine serum retinol-binding protein is included in the gel. An apparent molecular weight of approximately 21,000 distinguishes this serum protein from the retinol-binding proteins (molecular weight approximately 17,000) we find in retina\(^{7}\) and RPE. In addition, the retinol-binding protein from the two sources behaved similarly in that only retinol was able to effectively displace \(^{3}H\)retinol from the protein. Retinal, retinoic acid, retinyl acetate, and retinyl palmitate were all ineffective when added in a 100-fold molar excess. Similar properties were found for the retinol-binding protein from retina.\(^{18}\) It thus seems likely that the same or a similar binding protein is present in both retina and RPE.

Sonication and centrifugation of isolated outer segments yielded a supernatant containing retinol-binding protein. Approximately 10 percent of the total binder of the retina was present in the outer segments, suggesting that most of the binding protein is localized in other portions of the retina. As shown here and in a previous study,\(^{16}\) there is approximately a fivefold excess of retinoic acid-binding protein over retinol-binding protein in extracts of bovine retina. However, we failed to recover significant amounts of retinoic acid-binding protein in extracts derived from isolated outer segments. A definitive localization of these binding proteins within the retina awaits immunocytochemical studies.

The wet weight of the bovine RPE cell layer has been reported\(^{13}\) to be approximately 3 to 5 mg., which is about 1 percent of the wet weight of the retina. Yet the RPE wash derived from one eye contained more retinol-binding protein than found in the entire retina, emphasizing the relatively large amount of this protein present in the RPE. It is likely that the high concentration of this specialized protein in the RPE reflects its important role in the uptake and transport of retinol.

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REFERENCES

2. Heller, J.: Interactions of plasma retinol-binding protein with its receptor. Specific binding of bovine and human retinol-binding protein...

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