Copyright © Association for Research in Vision and Ophthalmology

Characterization of Pigment Epithelial Cell Plasma Membranes From Normal and Dystrophic Rats

Sharon C. Braunagel,* Daniel T. Organisciak, and Hih-Min Wang

Retinal pigment epithelial cell plasma membranes were isolated from the eyes of normal and RCS-dystrophic rats by binding glass microbeads to the intact pigment epithelial cell layer, removal of the bead-bound cells from the eyes and subsequent sucrose density gradient centrifugation. Plasma membranes were recovered from the gradients in identical yields and characterized by membrane marker enzymes, lipid analysis and SDS-polyacrylamide gel electrophoresis. Membrane purification by alkaline phosphodiesterase 1 and 5’nucleotidase activities averaged 8-fold for normal rats and 5.5 for the dystrophic rats. The ratio of cholesterol per microgram protein indicated 6 to 7-fold purification for both types of plasma membranes. Na⁺K⁺-ATPase in the normal and mutant rat plasma membranes was purified 5- and 3.5-fold, respectively, but the specific activities of both Na⁺K⁺-ATPase and 5’nucleotidase were higher in the dystrophic rat membranes than in normal. Subcellular organelle contamination was low and relatively uniform in both types of membranes, while opsin contamination was less than 1%. By electrophoretic analysis the plasma membrane proteins were similar, with 30–40 identifiable bands present in each membrane type. The plasma membranes both contain high levels of cholesterol, sphingomyelin and phosphatidylcholine and low levels of polyunsaturated fatty acids. However, the dystrophic rat membranes had significantly higher levels of docosahexaenoic acid than normal, and significantly lower levels of arachidonic acid. The differences in these plasma membrane fatty acids and in the membrane-bound enzymes may affect the ionic balance of the interphotoreceptor matrix or otherwise contribute to degenerative changes in dystrophic rat photoreceptors. Invest Ophthalmol Vis Sci 29:1066-1075, 1988

Effective photoreceptor cell rod outer segment (ROS) membrane turnover is normally accomplished by the phagocytic removal of the shed ROS tips by the retinal pigment epithelium (RPE). However, phagocytosis, which is light-entrained in normal rats,2 is impaired in the Royal College of Surgeons (RCS) strain.2-5 RCS retinal-dystrophic rats have been studied as a model for human retinal degenerations and to determine the effects of light and eye pigmentation on ROS development, turnover and vitamin A transport6-13 (for a review see ref. 14). It is now firmly established, following studies with allopheic offspring of normal and mutant rats13 and normal and mutant RPE cells in culture mixed with ROS,15,17 that the genetic defect in the RCS rat resides in the RPE. Recently, the phenotypic expression of the endocytotic defect has been further localized to the RPE plasma membrane, where ROS binding occurs, although ingestion is impaired.18,19

Other abnormalities in the RCS rat RPE plasma membrane also exist. For example, Na⁺K⁺-ATPase, which is normally localized in the apical membrane processes,20 redistributes in the plasma membrane during cellular tight junction breakdown.21 Based on studies with RPE cells maintained in tissue culture, Clark and Hall22 demonstrated the incomplete glycosylation of a high molecular weight plasma membrane protein in mutant cells. Alterations in the distribution of plasma membrane cholesterol23,24 and in the lipid composition of RPE cells from young dystrophic rats25 have also been reported. However, the RPE plasma membrane has not yet been isolated from rats and characterized by classical biochemical methods. Using a glass bead technique originally developed for the isolation of bovine plasma membranes,26 we now report the isolation and partial purification of RPE plasma membranes from normal and dystrophic rats. Our study shows that the plasma membranes from RCS rats contain higher activities of Na⁺K⁺-ATPase and 5’nucleotidase than normal,
and lipid abnormalities which may further impair RPE function in these animals.

Materials and Methods
Animals, Maintenance, Breeding
Long Evans hooded rats weighing 75–100 g were obtained from Harlan Industries (Indianapolis, IN) and maintained in a 5 ft cd cyclic light environment on Purina Rat Chow and water. RCS albino and pigmented rats, originally obtained from W. K. Noell, were bred and raised in our animal facility. On postnatal day 7 mothers with litters were moved from the cyclic light environment to darkness to slow the progression of the retinal degeneration. Dystrophic rats were used as available from the colony, and were between the ages of 29–34 days. Normals were from rats were used as available from the colony, and were between the ages of 29–34 days. Normals were from W. K. Noell, dystrophic rats and pigmented normals were used while both pigmented and albino mutant rats were available for lipid analysis. As determined for both the normal and mutant animals eye pigmentation had no effect on the individual determinations, but pigmented eyes were easier to manipulate during tissue preparation. All animals were dark-adapted for 18 hr before sacrifice in halothane-saturated chambers and all dissections were performed in dim red light. The use of animals in this investigation conformed to the ARVO Resolution on the Use of Animals in Research.

RPE Plasma Membrane Isolation
Following enucleation in dim red light, the cornea and lens were removed and the remaining eye cup inverted over the conical end of a 0.5 ml polyethylene microfuge tube (5 × 45 mm; Beckman, Inc., Fullerton, CA). The tube, with adherent eye cup, was then placed into a phosphate-buffered saline solution (pH 7.2) at 4°C, containing 2 mM phenylmethyl-sulfonyl-fluoride, 2.5 mM iodoacetamide and 0.2% EDTA (PBS-PIE). Although the adhesive forces holding the outer scleral surface to the plastic have not been studied, the eye tissue-microfuge tube preparation remains intact and floating during the subsequent 30 min period in buffer. This preparation also provides a convenient way to handle the small rat eye without disrupting the RPE cell layer. Next, under ambient light, the plastic tubes were placed over small glass rods and the retinas gently teased away from the RPE cell surface. To remove adhering ROS, the inverted eye cups were then soaked for an additional 2 hr in cold PBS-PIE. Next, the RPE cells were coated with tris-treated glass microbeads, 1–33 μm (Cataphote, Inc., Jackson, MS) by carefully turning the plastic tubes in a petri dish containing the beads.

The exposed surfaces of the glass beads were then coated with 0.1% silane and the RPE cells, with adherent beads, carefully brushed from the eye cups into 30 ml of PBS buffer. The RPE cell-bead preparation was then washed with PBS and treated with collagenase as described. Following a saline wash, a whole cell lysate (whole cell) was prepared by treating the cells with 2 ml ice-cold water. Cytoplasmic contaminants were removed with three saline washes and the bead-bound RPE plasma membranes were then suspended in 2 ml of 40% sucrose. To release plasma membranes from the beads the preparation was vortexed vigorously for 1 min. Next, a discontinuous gradient was prepared by layering the 40% sucrose with 1 ml 32%, 0.5 ml 28% sucrose and water to a final volume of 5 ml. All sucrose solutions were made in 1 mM imidazole buffer pH 7.4 containing 0.1 mM MgCl₂. The preparation was centrifuged at 104,000 g for 1 hr at 4°C and the plasma membranes collected from the 28% sucrose layer. The precipitated bead-membrane fraction (beads) was recovered and stored in liquid nitrogen. The isolated plasma membranes were then precipitated by mixing with 50 mg fresh glass beads, diluting the sucrose to less than 10% with water and centrifuging at 120,000 g for 60 min at 4°C. This last step facilitated the handling of the small quantities of RPE plasma membranes, which bind tightly to unsilanized glass. The precipitated plasma membrane-glass bead pellet was collected and normally stored in liquid nitrogen until use. In a typical experiment 60 eyes were used for enzyme analysis, while 240–300 eyes were used for the lipid determinations.

Membrane Purity and Composition
To determine plasma membrane purity, the following enzymatic markers were assayed: 5'nucleotidase, alkaline phosphodiesterase I and Na⁺K⁺-ATPase. Lysosomal contamination was measured by N-acetyl-β-D-glucosaminidase activity. Endoplasmic reticulum and mitochondrial contamination were measured by sulfatase C and cytochrome oxidase activity, respectively. RPE membrane marker enzyme measurements have previously been described in detail. All enzyme assays were run at protein concentrations for which a linear response was determined. With the exception of Na⁺K⁺-ATPase, no differences in enzymatic activity were detected when fresh or frozen membrane samples were used. As a result, Na⁺K⁺-ATPase determinations were performed on fresh RPE preparations. To determine ROS contamination opsin was measured with a radioimmunoassay previously described by Planter et al. Protein was determined by the method of Lowry et al, with bovine serum albumin as the standard.
Table 1. Purification and yield for RPE plasma membranes from normal rats

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Alkaline phosphodiesterase I</th>
<th>5’Nucleotidase</th>
<th>Na’K+-ATPase</th>
<th>Average protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg PNP/µg/hr</td>
<td>nmol Pi/µg/hr</td>
<td>nmol Pi/µg/hr</td>
<td>µg/eye</td>
</tr>
<tr>
<td>Whole cell</td>
<td>0.33 ± 0.13</td>
<td>0.81 ± 0.29</td>
<td>1.02 ± 0.19</td>
<td>56.7 ± 25.2</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>2.70 ± 1.38 [8.2]†</td>
<td>6.55 ± 2.49 [8.1]</td>
<td>4.92 ± 1.19 [4.8]</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Bead</td>
<td>0.40 ± 0.34 [1.2] (1.2)</td>
<td>1.36 ± 1.02 [1.7] (2.2)</td>
<td>1.38 ± 0.89 [1.4] (1.4)</td>
<td>9.0 ± 4.4</td>
</tr>
</tbody>
</table>

*Results are the mean ± SD for the number n of experiments listed.† Fold purity in brackets.‡ (Relative specific activity) is defined as the total activity in a fraction/average protein of the fraction. Whole cell activity is set to (1) for each enzyme. § ND: Not detectable.

Lipid Analysis

Whole cell, bead and plasma membrane fractions were extracted with chloroform:methanol 2:1 and washed according to Folch et al.29 Aliquots of the lipid extracts were used for the preparation of fatty acid methyl esters, thin layer chromatographic analysis of lipid composition and lipid phosphorus and cholesterol determinations. Techniques for the analysis of membrane lipids have been described.121326 Plasma membrane purity was determined from the ratio of cholesterol to protein in each fraction.

Gel Electrophoresis

The basic technique of Laemmli30 was used for SDS-polyacrylamide gel electrophoresis. A 4% stacking gel was used over a 10–15% gradient gel. Samples were incubated in 3% SDS, 1% β-mercaptoethanol, 0.05 M Tris-HCl (pH 6.8) and 15% glycerol for 60 min at 37°C. Following electrophoresis, the proteins were fixed and stained with 0.2% Coomassie blue. Gels were photographed and the negatives scanned with an LKB-2202 Ultrascan laser densitometer (LKB, Bromma, Sweden).

Results

Membrane Enzyme Marker Analysis

The purity of each plasma membrane preparation was determined by measuring the activities of two or three plasma membrane and subcellular marker enzymes. The combined results of 13 separate membrane preparations for normal rats are contained in Table 1. Based on the specific activities for alkaline phosphodiesterase I and 5’nucleotidase in the whole cell fraction, RPE plasma membranes from normal rats were 8.2- and 8.1-fold purified, respectively. Plasma membrane Na’K+-ATPase, however, was only 4.8-fold purified. The relative specific activities for the same enzymes were very similar: 7.8, 8.1 and 5.0 respectively. This indicates that a true purification of the plasma membranes has occurred. These membranes contained an average of 11%, 7% and 4% of the total alkaline phosphodiesterase I, 5’nucleotidase and Na’K+-ATPase activities, respectively. On the average 0.6 µg protein/eye was recovered in the plasma membrane fraction, or 1% of the total whole cell protein. The bead fraction, from which the plasma membranes were separated, contained about 16% of the protein found in the whole cell. By both specific activity and relative specific activity, the purity of the bead fraction (row 3) was intermediate between those of the whole cell and plasma membranes.

Contamination of the plasma membranes by RPE subcellular organelles or ROS is shown in row 5 of Table 1. The lysosomal enzyme N-acetyl-β-D-glucosaminidase was 10-fold lower in the plasma membranes than in the whole cell fraction. The mito-
Table 2. Purification of dystrophic rat RPE plasma membranes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Alkaline phosphodiesterase I</th>
<th>5'Nucleotidase</th>
<th>Na⁺K⁺-ATPase</th>
<th>Average protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg PNP/µg/hr n = 3</td>
<td>nmol Pi/µg/hr n = 4</td>
<td>nmol Pi/µg/hr n = 4</td>
<td>µg/eye n = 10</td>
</tr>
<tr>
<td>Whole cell</td>
<td>0.30 ± 0.11 (1)</td>
<td>1.48 ± 0.34 (1)</td>
<td>2.24 ± 0.28 (1)</td>
<td>55.3 ± 12.6</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>1.51 ± 0.42 [5.0]†</td>
<td>7.80 ± 1.33 [5.3]</td>
<td>7.26 ± 2.11 [3.2]</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Bead</td>
<td>0.24 ± 0.08 (0.8)</td>
<td>2.27 ± 1.06 [1.5]</td>
<td>1.74 ± 0.67 [0.8]</td>
<td>7.3 ± 3.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>N-acetyl-β-D-glucosaminidase</th>
<th>Sulfatase C</th>
<th>Cytochrome oxidase</th>
<th>Opsin ng/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg PNP/µg/hr n = 3</td>
<td>fluorescence units/µg/hr n = 3</td>
<td>µMO2/µg/min n = 1</td>
<td></td>
</tr>
<tr>
<td>Whole cell</td>
<td>0.38 ± 0.12 (1)</td>
<td>—</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>0.08 ± 0.02 [0.2]</td>
<td>(0.22)</td>
<td>ND§</td>
<td>9.0</td>
</tr>
<tr>
<td>Bead</td>
<td>0.25 ± 0.12 [0.6]</td>
<td>(0.6)</td>
<td>0.01 [0.5]</td>
<td>—</td>
</tr>
</tbody>
</table>

* Results are the mean ± SD for the number n of experiments listed. Fold purity in brackets.
† Specific activity is defined as the total activity in a fraction/total protein in the fraction. Whole cell activity is set to (1) for each enzyme.
§ ND: Not detectable.

Chondrial marker cytochrome oxidase was 30-fold lower and sulfatase C, an endoplasmic reticulum enzyme, was 5-fold lower than in whole cell. Opsin was 0.6 ng/µg protein in the RPE whole cell fraction, but was undetectable in the plasma membranes. For completeness contaminating enzyme activities for the bead fraction are also included in Table 1 (row 6). As measured for the plasma membrane markers, bead values were intermediate between the whole cell and plasma membrane activities. However, unlike the plasma membrane enzymes, subcellular marker activities in the bead fraction were higher than in the plasma membranes.

Enzyme purification data for the RPE plasma membranes of RCS-dystrophic rats are given in Table 2. As measured by alkaline phosphodiesterase I and 5'nucleotidase these plasma membranes were 5.0- and 5.3-fold purified from the whole cell plasma membranes. For completeness contaminating enzyme activities for the bead fraction are also included in Table 1 (row 6). As measured for the plasma membrane markers, bead values were intermediate between the whole cell and plasma membrane activities. However, unlike the plasma membrane enzymes, subcellular marker activities in the bead fraction were higher than in the plasma membranes.

The same marker enzyme activities in the dystrophic rat bead fraction (rows 3 and 6) resembled more closely those in the whole cell fraction than those in the plasma membranes. Protein yields for the whole cell, bead and plasma membrane fractions of the dystrophic rat RPE were nearly identical to the values found for normal rats (Table 1).

A comparison of the plasma membrane markers found in Tables 1 and 2 reveals that both 5'nucleotidase and Na⁺K⁺-ATPase activities in the dystrophic rat fractions were higher than normal. For the whole cell fractions the dystrophic rat values were nearly two times higher. In the dystrophic rat plasma membranes 5'nucleotidase and Na⁺K⁺-ATPase were also higher than in normal, despite the fact that the mutant rat membranes were not as pure as those from normal rats. On the other hand, whole cell-alkaline phosphodiesterase I activities were practically the same. Alkaline phosphodiesterase in both the plasma membrane and bead fractions from mutant rats was lower than normal.

To determine if the higher activity of Na⁺K⁺-ATPase in the dystrophic rat RPE was an artefact of isolation or a result of ROS contamination, paired experiments with age-matched normal and RCS rats were performed (Table 3). In experiments 1 and 2 the whole cell activities for dystrophic rat RPE were 2.0- and 1.7-fold higher than in normal. Experiment 3 is a...
Table 3. Na⁺K⁺-ATPase specific activities in normal and dystrophic rat RPE and ROS (nmol Pi/μg protein/hr*)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Whole cell</th>
<th>Plasma membranes</th>
<th>Rod outer segments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Dystrophic</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>1.14</td>
<td>2.28</td>
<td>3.72 [3.3]*</td>
</tr>
<tr>
<td>2</td>
<td>1.44</td>
<td>2.36</td>
<td>4.56 [3.2]</td>
</tr>
<tr>
<td>3*</td>
<td>—</td>
<td>2.44</td>
<td>—</td>
</tr>
<tr>
<td>4*</td>
<td>—</td>
<td>2.44</td>
<td>—</td>
</tr>
</tbody>
</table>

* Values are for individual membrane preparations for paired experiments with normal and dystrophic rat RPE or ROS.
† Fold purification.
‡ Values obtained from pigmented dystrophic rats. Experiments 1 and 2 were obtained from albino rats.

result obtained for pigmented dystrophic rats. As shown, whole cell Na⁺K⁺-ATPase activity for the pigmented and albino dystrophic rats (exps. 1 and 2) are nearly identical. Although not shown in Table 3, 5'nucleotidase activity in the pigmented dystrophic rat whole cell fraction was 1.0 and alkaline phosphodiesterase I activity was 0.30. The dystrophic rat RPE plasma membrane Na⁺K⁺-ATPase activity was also higher than normal. In albino dystrophic rats, the plasma membrane activities were 5.9 and 6.8 compared to 3.7 and 4.6 for the normals. For the preparation of pigmented dystrophic rats Na⁺K⁺-ATPase activity was 15.8, giving a 6.5-fold purification from the whole cell activity. This is in good agreement with the purification data for pigmented normals in Table 1. Furthermore, these membranes were 11.9- and 10.2-fold purified by 5'nucleotidase and alkaline phosphodiesterase I activities. Thus, for these mutant rat RPE membranes Na⁺K⁺-ATPase purification was 55-64% of the values for 5'nucleotidase and alkaline phosphodiesterase; this also agrees well with the respective purifications for the same enzymes in Tables 1 and 2 (59-64%).

As shown by the ROS data in Table 3, the higher Na⁺K⁺-ATPase activity in dystrophic rat RPE plasma membranes is not from the small (<1%) contamination by opsin-containing ROS. The activities for young normal and dystrophic rat ROS are similar and lower than measured in either the whole cell or plasma membrane fractions from dystrophic rats. Similarly, Na⁺K⁺-ATPase activity in photoreceptor cell debris from older dystrophic rats was only 2.18 nmol Pi/μg protein/hr. 5'nucleotidase activity in the ROS of normal and mutant rats was 3.32 and 3.02 nmol Pi/μg/hr; it was 2.16 in the debris from RCS rats. Taken together, these data suggest that the RPE membrane enzyme activities in albino and pigmented dystrophic rats are the same, and that the higher activities found in the plasma membranes of pigmented animals probably relates to the ease of tissue preparation rather than to a difference in endogenous activity.

Gel Electrophoresis

Figure 1 shows the SDS-gel electrophoretic protein profiles for the respective normal and dystrophic rat whole cell (lanes 1 and 2) and plasma membrane fractions (lanes 3 and 4). The Coomassie-stained gels are shown in panel A, and the densitometry profiles in panel B. In lanes 1 and 2 a number of bands ranging in molecular weights between 97 and 14 kDa are prominent. A number of higher molecular weight proteins are not as well stained, but in each case the same proteins were present in both the normal and dystrophic rat fractions. In the normal, three low molecular weight proteins (14–17 kDa) were more intensely stained, while the dystrophic rat whole cell fraction (lane 2) contained a doublet of about 95–97 kDa which stained more intensely than normal. A similar 95–97 kDa protein, and a protein of about 67 kDa, stained prominently in the dystrophic rat plasma membrane fraction (lane 4). Otherwise, the plasma membrane proteins from normal and dystrophic rats were very similar. By Coomassie staining or densitometry, 30–40 proteins were detectable, ranging in molecular weight from over 200 kDa down to about 30 kDa. Concanavalin A-conjugated HRP stained about 20 high molecular weight proteins in transblots of RPE plasma membranes (data not shown), confirming the presence of several glycoproteins of over 100 kDa.22

Analysis of Membrane Lipids

The lipid class compositions for the normal and dystrophic rat RPE whole cell, plasma membrane and bead fractions are contained in Table 4. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major lipids present in the RPE. Together they account for between 70–80% of the total lipid phosphorous present in all fractions. Comparing the whole cell lipid profiles, PC was about 45% and nearly the same, while PE was almost 29% in dystrophic RPE compared to 23% in the normal. Sphingomyelin (SPH) was present in moderate amounts in the normal and mutant RPE, 14% and 11% respec-
respectively, while both phosphatidylserine (PS) and phosphatidylinositol (PI) were lower (5–6%). The molar ratio of cholesterol to lipid phosphorus was 0.4–0.3 (normal vs. RCS) in the whole cell fractions.

Based on the ratio of cholesterol/microgram protein present in the two types of plasma membranes and their respective whole cell values, purification of the normal rat membranes was 6.7, while the dystrophic rat plasma membranes were 6.2-fold pure. These purifications are in good agreement with the values obtained by enzyme marker analysis (Tables 1 and 2). In most respects the lipid composition of the plasma membranes resembled those of the RPE cell. As expected for plasma membranes, however, SPH and cholesterol were higher than in the whole cell fractions. In both types of rat plasma membranes PC was practically the same as in the whole cell fractions. Moderate levels of PE were present in each, but for the dystrophic rat plasma membranes the decrease from whole cell PE levels was over 7 mol%. PI was present in lower concentrations (3.1–3.6%) in both plasma membrane types, while PS was between 4–7 mol%. Lysophosphatidylcholine (LPC) represented less than 2% of the lipid phosphorus in all fractions, while phosphatidylglycerol and phosphatidic acid each, were less than 1.0% (data not shown). The molar ratio of cholesterol to lipid phosphorus was 2–3-fold higher in the plasma membranes than in whole cell, but similar in both the normal and mutant rat membranes.

The lipids of the normal and mutant rat bead fractions were also similar. However, in comparison to
the whole cell and plasma membrane fractions, PC in the bead fractions was higher, almost 55%, while SPH was much lower. Overall, the bead fractions were not purified from the whole cell lipids and the cholesterol:phosphate ratio was only slightly higher than for the RPE cells.

The fatty acid profiles for the normal and dystrophic rat retinal pigment epithelium are contained in Table 5. In the whole cell fractions the major saturated fatty acids were palmitic (16:0) and stearic (18:0) acids, which together represented 45–50% of the total. Palmitic acid was almost 8% higher in the dystrophic rat RPE, while 18:0 was nearly equivalent in the two whole cell fractions. The major unsaturates were arachidonic acid (20:4), oleic acid (18:1) and docosahexaenoic acid (22:6). Although the levels of 20:4 and 18:1 were lower in dystrophic rat whole cell lipids, 22:6 was two times higher than normal (13.2 vs. 5.9 mol%).

In the RPE plasma membranes of normal rat 16:0 was 33 mol%; it was 38% in the plasma membranes from dystrophic rats. In both types of plasma membranes 18:0 levels were similar to those found for whole cell. Arachidonic acid was substantially lower in both types of membranes, but it was only 5.4 mol% in the dystrophic rat samples compared to 12.8% in normal. Similarly, 22:6 was about 50% lower in the plasma membranes than in the respective whole cell fractions. In the normal, 22:6 was less than 3%, however, it was 6 mol% in the dystrophic rat RPE plasma membranes. A comparison of the fatty acid compositions of the two bead fractions shows that they resemble the whole cell fatty acids more closely than those of the plasma membranes. At the same time, the levels of many of the major fatty acids (eg, 16:0, 18:0, 20:4, 22:6) in the beads were intermediate between those of the other two fractions.

Discussion

This study shows that the RPE plasma membrane of RCS rats is characterized by higher than normal specific activities of the marker enzymes Na+K+-ATPase and 5’nucleotidase and by abnormalities in the contents of the 22:6 and 20:4 fatty acids. The same enzyme and lipid differences were present in the whole cell fractions of albino and pigmented RCS rats, suggesting that the plasma membrane abnormalities are not an artefact of isolation. Several additional lines of evidence also support these findings. First, not all plasma membrane enzymes were affected to the same extent. Alkaline phosphodiesterase I activity was the same in the whole cell fractions of both normal and mutant rats. Despite the different purifications of the normal and dystrophic rat plasma membranes (8- and 5-fold, respectively), if phosphodiesterase activities are calculated at equivalent membrane purities then they would also be very similar. The same applies to the two bead fractions from which the plasma membranes were isolated (Tables 1 and 2). Second, the protein yields for the various normal and dystrophic rat membrane fractions were similar. This indicates that differential membrane losses probably did not occur during the isolations and that plasma membrane release from the beads was nearly equivalent. Based on a consideration of the protein yields and enzyme recovery data we estimate that between 10–25% of the total theoretical plasma membrane protein was recovered in our samples. Thus, it is unlikely that a small unrepresentative fraction of the RPE plasma membranes was isolated.

<table>
<thead>
<tr>
<th>Table 5. Fatty acid composition of normal and dystrophic rat retinal pigment epithelium* (Mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty Acid</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>14:0</td>
</tr>
<tr>
<td>16:0</td>
</tr>
<tr>
<td>16:1</td>
</tr>
<tr>
<td>18:0</td>
</tr>
<tr>
<td>18:1</td>
</tr>
<tr>
<td>18:2</td>
</tr>
<tr>
<td>20:4</td>
</tr>
<tr>
<td>22:4</td>
</tr>
<tr>
<td>22:5ω6</td>
</tr>
<tr>
<td>22:5ω3</td>
</tr>
<tr>
<td>22:6</td>
</tr>
</tbody>
</table>

* Results are the mean ± SD for the number of separate preparations in parenthesis.
† Abbreviations stand for: chain length: number of double bonds; 22:5ω6 or ω3 identify the first double bond from the methyl end for this pair of equal chain length fatty acids.
Furthermore, contamination by subcellular organelles was relatively low and consistent between the two RPE plasma membrane types. Finally, Na\(^+\)K\(^-\)-ATPase and 5\'nucleotidase activities in the ROS or debris from dystrophic rats were lower than in the RPE fractions (Table 3). Thus, at a contamination of less than 1% opsin in the dystrophic rat RPE plasma membranes, an increase in specific activity of these marker enzymes from ROS would not be expected.

One obvious possibility to explain the elevated activities of plasma membrane enzymes in our dystrophic rat preparations is that they may contain more apical RPE membranes than normal. Certainly, judging by the SDS-gel protein profiles in Figure 1, the more intense staining of bands in the 95–97 kDa region of the dystrophic rat lanes indicates that these proteins are present in higher concentrations than normal. In addition, although we have not further identified the 97 kDa protein present in our gels, the catalytic subunit of Na\(^+\)K\(^-\)-ATPase has a molecular weight of 97 kDa. However, other evidence argues against a preferential purification of apical processes from the dystrophic rat plasma membranes. In both types of RPE the known apical membrane marker Na\(^+\)K\(^-\)-ATPase was purified to about 60% of the other plasma membrane enzymes. Furthermore, the activity of 5\'nucleotidase, an enzyme that appears to be localized over both the apical and basal surfaces of the RPE cell, was also elevated in the dystrophic rat fractions. This suggests that our plasma membranes contain both the apical and basal components of the RPE cell surface, and that the adherence of apical membranes to the glass beads was equivalent and somewhat greater than for the basolateral membranes. Clearly, measurements of a known basal membrane marker, such as the receptor for serum retinol binding protein, would allow a better estimate of the apical and basolateral membrane content of our preparations.

A second possibility is based on the finding that Na\(^+\)K\(^-\)-ATPase redistributes in the RPE plasma membrane of dystrophic rats during the breakdown of tight junctional complexes. As such Na\(^+\)K\(^-\)-ATPase would also be expected to be present in basolateral membranes, or in the additional apical plasma membrane invaginations seen in the RPE of young mutant rats. Changes in the lipid composition or fatty acids of the RPE membrane might also facilitate the lateral migration of membrane bound enzymes. Previous work shows that the binding of filipin and digitonin by cholesterol and lipid composition are abnormal in young dystrophic rat RPE. In addition, Na\(^+\)K\(^-\)-ATPase is more active in fluid membrane regions than in highly ordered regions, or in membranes containing high concentrations of cholesterol. Na\(^+\)K\(^-\)-ATPase also reportedly binds several hundred phospholipid molecules and is stimulated by PS. While our data suggest that major differences in the phospholipid head groups and cholesterol contents of the normal and dystrophic rat RPE plasma membranes do not exist, the regional localization of these lipids and their respective fatty acid compositions is currently unknown.

We did find remarkable differences in two major polyunsaturated fatty acids in the dystrophic rat RPE plasma membranes; 22:6 was higher than normal, while 20:4 was lower than normal (Table 5). These lipid differences appear to be a result of the lack of phagocytosis in RCS rats. Based on a consideration of the opsin contents in the various fractions, a small amount of extracellular photoreceptor cell debris may adhere to or have been trapped in the plasma membranes of the dystrophic rats. Whether this amount of contamination is sufficient to account for the fatty acid changes or not, the close association of the RPE and the photoreceptor debris in RCS animals suggests that lipid exchange between the two membranes can occur. For example, it is known that debris contains different levels of 22:6, PE and cholesterol than normal rat ROS membranes. In addition to the possible effect of lipid exchange (22:6) on Na\(^+\)K\(^-\)-ATPase activity in the RPE plasma membrane, the level of 20:4 was much lower in all membrane fractions from mutant rats. In very young dystrophic rats 20:4 appears to be normal in the RPE, while in debris from old dystrophic rats it is elevated. Whatever the reason for the loss of 20:4 from the dystrophic rat RPE, this evidence suggests that arachidonic acid metabolism in the mutant cells may be abnormal.

Finally, the elevation of RPE enzyme activity in RCS rats may be an attempt to compensate for the primary genetic defect by a process of up regulation in existing RPE membranes, or by the actual proliferation of the RPE apical microvilli. Up regulation of Na\(^+\)K\(^-\)-ATPase has previously been demonstrated in the epithelium of kidney cortical collecting tubule and in the epithelium of colon. Furthermore, Miller et al concluded that the electrogenic nature of Na\(^+\)K\(^-\)-ATPase and its location in the apical RPE membrane are likely to affect the transport of ions, fluids and metabolites across the RPE cell layer. Therefore, an increase in RPE membrane enzyme activity, for whatever reason, could alter the ionic milieu of the interphotoreceptor cell matrix. In addition, 5\'nucleotidase, through the release of adenosine, may play a role in the regulation of Na\(^+\) ion permeability in ROS. Recently, Irons has shown that a
Mn\(^{2+}\)-dependent pyrimidine 5' nucleotidase redistributes between RPE and ROS during shedding of the rod tips. Although this enzyme and the 5' nucleotidase activity measured in our study are not the same, the accumulation of abnormally high levels of otherwise normal enzyme activities in dystrophic RPE could affect these cells as well as the interphotoreceptor matrix. The added burden of the diffusional barrier imposed by the accumulation of photoreceptor cell debris as dystrophic rats age\(^1\) may also explain some of the pathological changes seen in the RPE of older animals.

The mechanism by which the well described ROS degeneration occurs in RCS rats remains an open question. However, Mullen and LaVail's\(^15\) observation that patches of normal RPE in chimeric rat eyes somehow modifies the destructive process in adjacent ROS facing dystrophic RPE cells lends support to the idea that a diffusable process is involved in the rod cell degeneration (see also ref. 14). In this regard it is interesting that retinol esterification, a process thought to be associated with the RPE plasma membrane,\(^42\) is abnormally low in dystrophic rat RPE.\(^43\) Furthermore, light exposure\(^9\)\(^10\) and the maintenance of RCS rats on a vitamin A-deficient diet\(^11\) each accelerate the destruction of ROS. The extent to which vitamin A metabolism is associated with the degenerative process in dystrophic rat photoreceptor cells or RPE remains to be determined.

Key words: retinal pigment epithelium, rat, plasma membranes, retinal dystrophy

Acknowledgments

We thank Dr. James Plantner for the opsin RIA analysis and Dr. I. M. Leffak for the laser densitometry.

References