

High Levels of E-/P-Cadherin: Correlation with Decreased Apical Polarity of Na/K ATPase in Bovine RPE Cells In Situ

Janice M. Burke,^{1,2} Feng Cao,¹ and Pamela E. Irving¹

PURPOSE. The adherens junction protein E-cadherin induces a basolateral polarity of Na/K ATPase in most epithelial cells that express it, whereas in retinal pigment epithelium (RPE) cells, Na/K ATPase is largely apical. The purpose of this study was to determine whether the distribution of Na/K ATPase differs in RPE cells in situ, that differ in levels of junctional E-cadherin.

METHODS. Bovine RPE cells in situ were immunostained with an E-cadherin antibody (which has some cross-reactivity with the closely related epithelial cadherin P-cadherin), and RPE cells with different levels of junctional stain were identified. RPE cells with low and high E-/P-cadherin were costained in various combinations with Na/K ATPase and interacting proteins of the membrane cytoskeleton (ankyrin, fodrin, and actin) and analyzed by confocal imaging.

RESULTS. Individual RPE cells within the same monolayer differed in amount of Na/K ATPase, with a lower frequency of high expressing cells in the area centralis. High expressing Na/K ATPase cells were found among cells with both low and high E-/P-cadherin levels. In cells with low E-/P-cadherin, Na/K ATPase localized to apical microvilli, whereas in high E-/P-cadherin cells, Na/K ATPase was on basolateral surfaces in addition to microvilli. Actin staining showed that microvillar domains were smaller and that lateral membrane domains were taller in high E-/P-cadherin cells. In high but not low E-/P-cadherin cells, ankyrin and fodrin levels varied among cells, with a subset of cells showing distinctly higher expression. Both ankyrin and fodrin had complex subcellular distribution patterns, although they tended to be enriched basal to rather than apical to the adherens junction. Cells with high Na/K ATPase did not necessarily have commensurately higher levels of ankyrin or fodrin. Where both Na/K ATPase and ankyrin were high, they codistributed weakly in apical microvilli but more prominently on the basal cell surface.

CONCLUSIONS. Within the same RPE monolayer, the polarity of Na/K ATPase differs among cells, with a more basal polarity found in cells with high levels of junctional E-/P-cadherin. The increased basal Na/K ATPase was due to a combination of a smaller microvillar domain, a taller lateral domain, and more basolateral staining for Na/K ATPase, perhaps because of an enrichment of a basal ankyrin-fodrin membrane cytoskeleton with which Na/K ATPase is known to associate. (*Invest Ophthalmol Vis Sci.* 2000;41:1945-1952)

The calcium-dependent adhesion protein E-cadherin forms cell-cell attachments at the zonula adherens junction of most monolayer epithelial cells. Retinal pigment epithelial (RPE) cells were believed to be an exception and to express N-cadherin rather than E-cadherin.¹⁻⁶ We recently observed, however, that E-cadherin is found in adult human RPE cells in situ and in postconfluent human RPE cell cultures.⁷ P-cadherin, an epithelial cadherin that is coexpressed with E-cadherin in many cells, is also expressed in human RPE,⁷ and

the avian homologue of P-cadherin (B-cadherin)⁸ has been found in chick RPE cells.⁹ The presence of E-cadherin in RPE cells is of particular interest because E-cadherin is a morphoregulatory protein, capable of inducing a specific polarity of cell surface molecules in the cells that express it. An example is the epithelial cell line MDCK in which Na/K ATPase and its associated ankyrin-fodrin-actin membrane cytoskeleton are enriched on lateral membranes, basal to the adherens junction. This distribution appears to be induced by the formation of E-cadherin adhesions.^{10,11}

In contrast, in RPE cells in situ, Na/K ATPase is polarized predominantly to apical microvilli,¹²⁻¹⁸ a distribution that appears inconsistent with the expression of E-cadherin in the tissue. Furthermore, when a cultured rat RPE cell line was transfected to express E-cadherin, Na/K ATPase developed a basal polarity,⁴ indicating that E-cadherin is capable of inducing a basal distribution even in a cell type in which the pump is normally apical. The apparent inconsistency of the observations that E-cadherin is expressed in RPE cells in situ, where the pump is reportedly apical, led us to reevaluate Na/K ATPase distribution in RPE cells within the eye. In the RPE

From the Departments of ¹Ophthalmology and Cell Biology, ²Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin.

Supported by Grants R01-EY10832 (JMB) and P30-EY01931 from the National Eye Institute; and by unrestricted grants from the Posner Foundation (Milwaukee, Wisconsin) and Research to Prevent Blindness (New York, New York). JMB is the recipient of a Senior Scientific Investigator Award from Research to Prevent Blindness.

Submitted for publication November 15, 1999; revised January 18, 2000; accepted January 31, 2000.

Commercial relationships policy: N.

Corresponding author: Janice M. Burke, The Eye Institute, 925 North 87th Street, Milwaukee, WI 53226-4812. jburke@mcw.edu

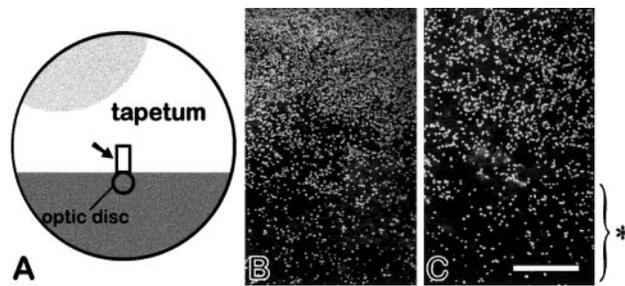


FIGURE 1. Diagram of the posterior pole of the bovine eye (A), accompanied by low-power immunofluorescence micrographs of the RPE monolayer from two animals, immunostained for Na/K ATPase (B, C). The tissue pieces used for immunostaining were taken from a region just superior to the optic disc (indicated by the *rectangle* and *arrow* in A). The *small white dots* in (B) and (C) are individual RPE cells with high levels of Na/K ATPase. The frequency of cells with high Na/K ATPase is higher in eye (B) than in eye (C). In both eyes, there are fewer high Na/K ATPase cells in the posterior region (*) than in the more peripheral region of the tissue piece. Scale bar, 400 μ m.

monolayer, cadherin expression levels vary among fields of cells, both in situ and in vitro.⁷ We took advantage of this naturally occurring variation to compare protein distributions in RPE cells with high and low E-cadherin levels, focusing on Na/K ATPase and proteins with which it is linked: ankyrin, fodrin, and actin. Bovine RPE cells were used because cells from the tapetal region of this species have relatively few melanin and lipofuscin granules, which obscure detailed visualization of cytoplasmic and cell surface proteins.

METHODS

Tissue Preparation

Eyes from adult cattle were obtained approximately 3 to 5 hours postmortem; approximately 400 eyes were used for this study. The retina was removed and the eyecup was filled with fixative (phosphate-buffered 3% paraformaldehyde, 4°C). After fixation (1–3 hours), pieces of RPE with underlying choroid (approximately 5 × 10 mm rectangles) were dissected from the tapetal region of the superior retina. In early studies, tissue was taken from throughout the tapetum, immunostained for various antigens and combinations of antigens (indicated in the next section), and examined and photographed by epifluorescence microscopy. After consistent morphologic features were identified, 85 eyes were prepared for confocal imaging, focusing on a constant site just superior to the optic disc (Fig. 1). All proteins were examined in a minimum of 10 different eyes, and the images that are shown are representative of multiple microscope fields.

For some eyes, the retina was not separated from the RPE. Rather, the choroid and RPE with the retina attached were frozen, and 10- μ m-thick cross sections were cut.

Fluorescence Microscopy and Confocal Imaging

Tissue pieces or frozen sections containing the RPE monolayer were permeabilized by incubation in 0.5% Triton and immunostained with one or more of the following primary antibodies:

monoclonal anti-E-cadherin (clone 36; Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-ankyrin (directed against chicken erythrocytes; Calbiochem, La Jolla, CA), rabbit polyclonal anti-fodrin/spectrin (Sigma, St. Louis, MO), and chicken polyclonal anti-Na/K ATPase ($\alpha 1\beta 1$; Cortex Biochem, San Leandro, CA). Antibody dilutions and times of incubation were empirically determined. Appropriate FITC (fluorescein)-, TRITC (rhodamine)-, or LRSC (lissamine rhodamine)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). To visualize F-actin, tissues were stained with fluorescein- or rhodamine-conjugated phalloidin (Sigma). Frozen sections were counterstained with propidium iodide to stain nuclei. The clone 36 E-cadherin antibody gives a strong immunostaining signal in bovine RPE and was therefore used for the experiments reported here. Weaker junctional staining (not shown) was also obtained with SHE78-7 and HEC1-1 monoclonal E-cadherin antibodies (Zymed Laboratories, San Francisco, CA). The clone 36 E-cadherin antibody exhibits some cross-reactivity with P-cadherin, which is coexpressed with E-cadherin in many epithelial cells, including the RPE⁷; we have confirmed by immunoblot analysis that P-cadherin is found in bovine RPE (not shown). Because of the cross-reactivity of the clone 36 E-cadherin antibody, cells stained here are designated as E/P-cadherin positive.

Immunostained preparations were examined and photographed with an epifluorescence microscope or a Bio-Rad MRC-600 confocal microscope (Bio-Rad Laboratories, Hercules, CA). For the latter, serial 0.2- or 0.5- μ m-thick sections were collected through the RPE monolayer to generate cross-sectional Z-scans and to produce scans from which composite en face images could be reconstructed. Because the tissue pieces are not completely flat and because RPE cell height varies (from approximately 8–15 μ m), 10 to 30 scans were collected above and below the monolayer to assure that the full thicknesses of all cells in the monolayer were imaged. For some purposes a subset of scans is shown, and the scan numbers are reported in the figure legends. The most apical scan was designated as scan 1; it is the scan in which a microvillar marker (e.g., Na/K ATPase) first appears in the tallest cell in the field. The scan number of the basal cell surface is also reported, using markers that are indicated in the legends. Because the RPE has basal infoldings and because tissue pieces are not flat, basal antigens occur in several scans (10 or more). The estimate for the scan number of the basal cell surface is therefore approximate and not identical for all cells in the field. Comparisons of protein distributions in low and high E/P-cadherin fields were made on the same tissue pieces or on tissue samples that were stained at the same time and imaged using identical settings. Quantitative comparisons of the size of different subcellular domains were made only on cells within the same tissue pieces. Bovine RPE cells contain two classes of autofluorescent inclusions: small irregular granules in the apical cytoplasm and base of microvilli and larger lipofuscin/melanolipofuscin granules in the plane of the adherens junction. These are more prominent when TRITC is used for the detection fluor. For imaging of immunostained samples, fields of cells were selected that were relatively deficient in these granules. For some purposes, the position of the granules within the cells was used as an endogenous marker of the apical cytoplasm.

RESULTS

Gross Distribution of Na/K ATPase in the RPE Cells of Bovine Eyes

The amount of Na/K ATPase varied markedly among individual RPE cells, with adjacent single cells in the monolayer showing prominent immunostaining differences. A subset of cells with distinctly higher levels of Na/K ATPase can be detected on low-magnification images of the monolayer (Figs. 1B, 1C). The frequency of these cells differed among eyes, but in all eyes they showed a consistent topographical pattern, being less numerous in a horizontal band superior to the optic disc, which corresponds to the area centralis. The tissue used for morphologic analysis was taken from within this region, immediately superior to the disc (Fig. 1A).

To confirm that the cell-cell differences in Na/K ATPase immunostaining were not due to damage to the RPE during retinal dissection, frozen sections were prepared from specimens in which the retina was not removed. Adjacent RPE cells with high and low levels of Na/K ATPase immunostaining were also seen in these cross sections (Fig. 2A). Note that the isoform of Na/K ATPase recognized by the antibody ($\alpha 1\beta 1$) was not found in the outer sensory retina but was found in the endothelium of the choriocapillaris (Fig. 2A).

Additional confirmation of the integrity of RPE cells with different levels of sodium pump was obtained by confocal imaging of cells costained for Na/K ATPase and actin (Fig. 2B). Because actin forms the core of apical microvilli and a prominent circumferential bundle in the apical cytoplasm of all cells, it serves as a marker to show that the apices of RPE cells with low levels of Na/K ATPase are intact and indistinguishable from cells with high Na/K ATPase staining (Fig. 2B, items a and b).

Comparison of RPE Cells with Different Levels of E-/P-Cadherin: Na/K ATPase, Ankyrin, and Fodrin

The amount of junctional E-/P-cadherin detected by immunostaining varied among bovine eyes, and among fields of RPE cells within the same monolayer. Using identical confocal settings, E-/P-cadherin staining varied from undetectable (or nearly so) in some fields of cells to a prominent junctional band in other fields (Figs. 3A, 3B).

Costaining showed no correlation between amounts of Na/K ATPase and amounts of junctional E-/P-cadherin; the scattered cells with high levels of Na/K ATPase were found in fields of cells regardless of level of cadherin staining (Figs. 3 through 5). Although amounts of Na/K ATPase did not differ, the subcellular distribution of Na/K ATPase did differ in cells with different levels of E-/P-cadherin. In low E-/P-cadherin cells, Na/K ATPase was predominantly on apical microvilli (Figs. 3E, E'; 4C1 through 4C3; 5A, A'), which were frequently very long (Fig. 3E, E'). In high E-/P-cadherin cells, Na/K ATPase was on lateral and basal cell surfaces (Figs. 3F, F'; 4C1 through 4C3; 5B, B') in addition to apical microvilli, which were shorter than in low E-/P-cadherin cells (compare Figs. 3E' and 3F', or Figs. 5A' and 5B').

Differences in Na/K ATPase distribution could be seen in high Na/K ATPase cells that were found in adjacent low and high E-/P-cadherin regions within the same microscope field (Fig. 4). Examination of images at different planes in the apico-basal axis showed distinct Na/K ATPase-stained puncta in the perinuclear cytoplasm of high but not low E-/P-cadherin

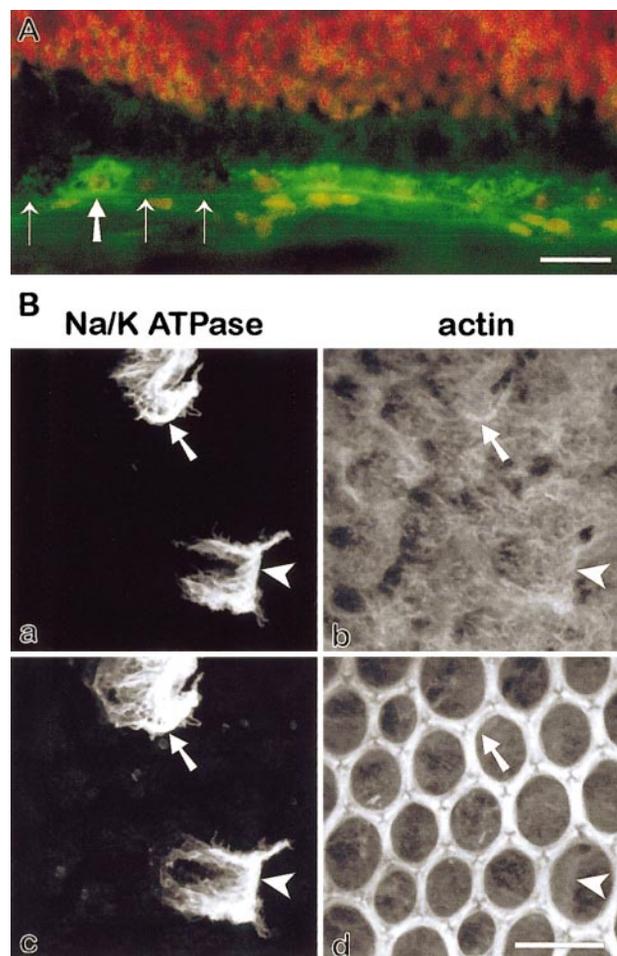


FIGURE 2. (A) Frozen section of the outer retina, RPE, and choroid immunostained for Na/K ATPase (fluorescein) showing prominent staining in some RPE cells (e.g., *thick arrow*) adjacent to cells with low staining (*thin arrows*). Nuclei are counterstained with propidium iodide. (B) Confocal images of the RPE monolayer dually stained for Na/K ATPase and actin. The upper images (a, b) show scans in the plane of the apical microvilli where actin stains the microvillar core (scans 1-10, 0.5- μ m scans). The cell base appears in scan 30. The lower images (c, d) are composites of all scans, through the entire thickness of the monolayer. For actin, the circumferential microfilament bundles are the most prominent staining pattern in the composite image. *Arrows* and *arrowheads* indicate the same positions in all images. Scale bars, 20 μ m.

RPE cells (Fig. 4C2). Examination of cells within the same field also showed apparent differences in the height of the lateral membrane of the cell body in cells with different cadherin levels. A confocal scan taken through a middle plane of the monolayer (Fig. 4C2) passes through the base of the microvilli in low E-/P-cadherin cells, which have long microvilli and short lateral membranes. The scan at the same plane through a nearby high E-/P-cadherin cell passes through the nuclear region because the cell has a taller lateral membrane and short microvilli. Height differences between microvillar and lateral membrane domains of cells with different cadherin levels were examined further by comparing cells stained for actin (see Fig. 6).

For ankyrin, expression levels appeared to correlate with levels of E-/P-cadherin. In high E-/P-cadherin cell fields (but not

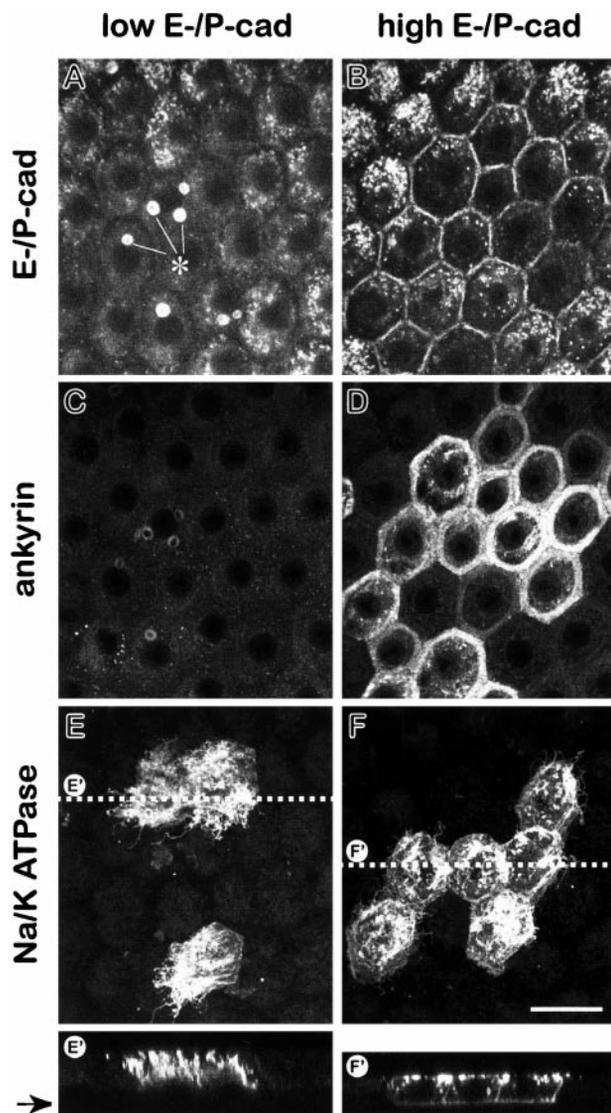


FIGURE 3. Confocal images of low and high E/P-cadherin fields, each triple stained for E/P-cadherin (A, B), ankyrin (C, D), and Na/K ATPase (E, F). For E-cadherin and ankyrin, selected $0.2 \mu\text{m}$ scans are shown for the high E/P-cadherin region taken from a plane encompassing the adherens junction and peripheral ankyrin ring (B and D, scans 23–29). Scan numbering is based on Na/K ATPase staining, where scan 1 is the apex of the tallest cell and scan 54 is the cell base. Selected scans at a comparable position within the cells are also shown for the low E/P-cadherin region (A and C, scans 32–38). Scan numbering for low E/P-cadherin cells is based on Na/K ATPase for the cell apex (scan 1) and ankyrin for the cell base (scan 55). The cytoplasmic fluorescence in the cadherin images is due to autofluorescent inclusions and lipofuscin granules; lipofuscin/melanolipofuscin granules are indicated by an *asterisk* in (A). For Na/K ATPase, cross-sectional Z-scans are shown; the positions of the Z-scans (E', F') are indicated by the *dotted lines* on the composite images (E, F, respectively). The *arrow* at the left of the Z-scans indicates the plane of the cell base. Scale bar, $20 \mu\text{m}$.

low fields) a subset of cells had markedly higher levels of ankyrin, much of which was distributed in a distinct peripheral ring (Figs. 3D, 4B). High ankyrin cells did not necessarily have high Na/K ATPase (and vice versa; see also Fig. 8).

Fodrin showed highly complex distributions among and within RPE cells in situ (Fig. 5). In cells with low E/P-cadherin,

levels of fodrin were relatively homogeneous among cells, and the protein was distributed throughout the cytoplasm, with somewhat higher staining between the nucleus and lateral cell borders, basal to the adherens junction (Fig. 5C,C'). Microvillar staining for fodrin was detectable but not prominent and was not higher in cells with high levels of microvillar Na/K ATPase (Fig. 5C'). In fields of cells with higher E/P-cadherin levels, fodrin staining intensity was more variable among cells (Fig. 5D). There were groups of cells with distinctly higher levels of fodrin, and in some of these cells fodrin distributed in a discrete, thin apicolateral ring (Fig. 5D, inset), in addition to an enrichment of the more diffuse basolateral cytoplasmic staining.

Comparison of RPE Cells with Different Levels of E-/P-Cadherin: Size of Microvillar and Lateral Membrane Domains

Microvillar size in RPE cells with different levels of E/P-cadherin was examined using specimens in which microvilli were visualized by staining their actin core (Fig. 6). Actin staining was also used to estimate the height of the lateral membrane domain of the cell body using the circumferential actin bundle as a marker. The actin bundle is associated with the adherens junction, which demarcates apical and basolateral membranes. The distance from the circumferential bundle to the cell base, therefore, provides a measure of the height of the lateral border of the cell body. Using actin staining, differences in height of both the microvillar and the lateral domains were seen in adjacent fields of RPE cells with different levels of

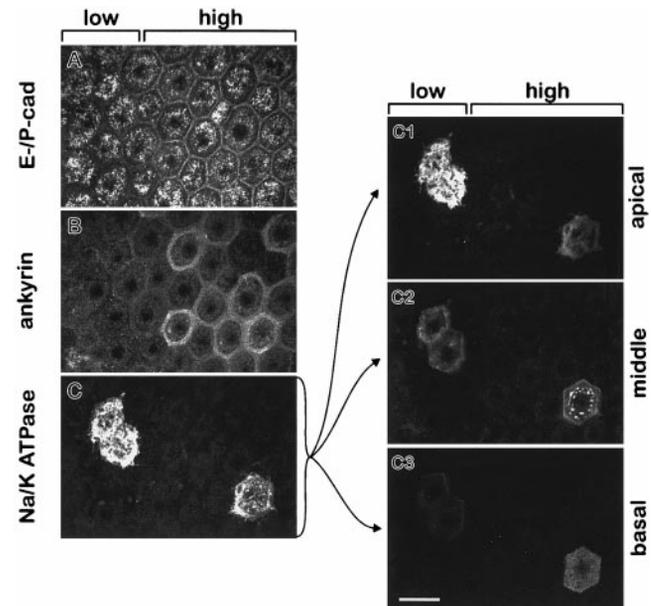


FIGURE 4. Composite confocal images of adjacent low and high E/P-cadherin (E/P-cad) regions in the same microscope field, stained for E/P-cadherin (A), ankyrin (B), and Na/K ATPase (C). For Na/K ATPase, selected individual $0.2 \mu\text{m}$ scans are also shown in the apical (C1, scan 13), middle (C2, scan 28), and basal (C3, scan 38) planes of the cells. For scan numbering, scan 1 is the apex of the tallest cell, and scan 46 is the cell base. Apical microvillar staining is greater for low E/P-cadherin cells (C1), and basal staining is greater for high E/P-cadherin cells (C3). In high E/P-cadherin cells, perinuclear structures prominently stained for Na/K ATPase are seen (C2). The nucleus appears as an *unstained hole* in the central cytoplasm. Scale bar, $20 \mu\text{m}$.

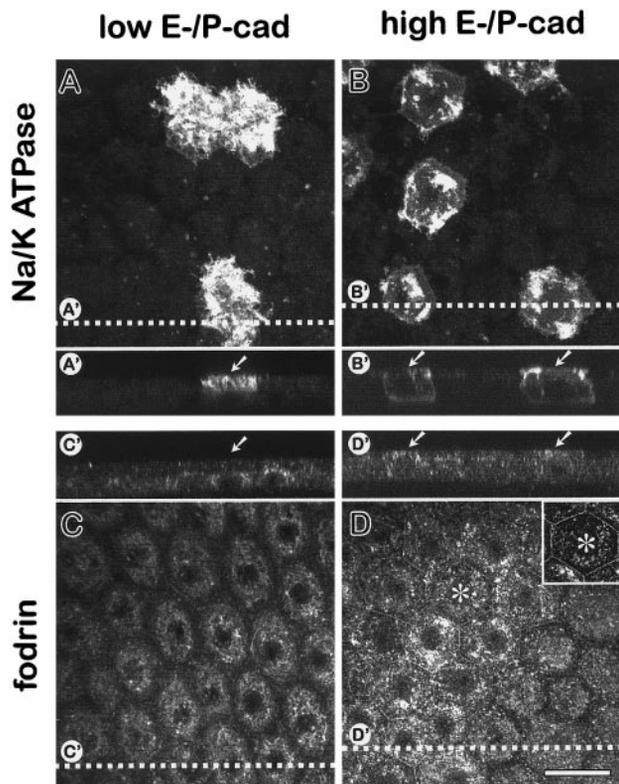


FIGURE 5. Confocal images of low and high E-/P-cadherin regions (E-/P-cad; cadherin staining not shown), each costained for Na/K ATPase and fodrin. Composite images and cross-sectional Z-scans are shown; the position of the Z-scans (A' through D'), which pass through high Na/K ATPase cells, are indicated by the dotted lines on their respective composite images. Fodrin immunostaining was more variable among cells in high E-/P-cadherin regions, where a thin pericellular fodrin band is sometimes seen (D, inset). The inset shows a single 0.2 μm (scan 27) in the plane of the pericellular fodrin band for the cell indicated by the asterisk in (D). (Scan numbering is based on Na/K ATPase staining where scan 1 is the apex of the tallest cell and scan 66 is the cell base.) Na/K ATPase is largely microvillar in low E-/P-cadherin regions (A') and on all cell surfaces in high E-/P-cadherin regions (B'). Fodrin stained microvilli, but microvillar staining is not prominent and not greater in microvilli with high Na/K ATPase (arrows in A' [Na/K ATPase] and C' [fodrin]). The cells with high Na/K ATPase in the high E-/P-cadherin field are also indicated by arrows (B' [Na/K ATPase] and D' [fodrin]). Scale bar, 20 μm .

E-/P-cadherin (Fig. 6). Confocal Z-scans (Fig. 6B') showed that cells with low levels of E-/P-cadherin have relatively long microvilli and short cell bodies compared with cells with higher levels of E-/P-cadherin. Sequential en face confocal scans through adjacent low and high E-/P-cadherin cells from the plane of their adherens junctions to the cell base could be used to estimate the differences in height of lateral membranes of the cell body. The plane of the adherens junctions was approximately 2 μm higher in cells with higher amounts of E-/P-cadherin, reflecting their taller cell bodies. The different heights of the cell bodies could also be shown using endogenous autofluorescent granules rather than the actin bundle as a marker of the cell apex (Fig. 6A,A'). The granules are distributed in the apical cytoplasm and in the base of microvilli; in cells with high E-/P-cadherin the distance from the granules to the cell base was visibly greater than in low E-/P-cadherin cells (Fig. 6A').

As shown in Figures 3D and 4B, a subset of high E-/P-cadherin cells also has high levels of ankyrin, with ankyrin forming a prominent peripheral ring. Among high E-/P-cadherin cells, cells with high levels of ankyrin have an even taller lateral cell domain than adjacent cells (Fig. 7). As shown, the distance from the ankyrin ring to the cell base is greater at the borders between adjacent high and low ankyrin cells than at the borders between adjacent high ankyrin cells (Fig. 7A'). Using the actin bundle-to-base as a measure of basal cell height (as in Fig. 5) in cells costained with actin and ankyrin, high ankyrin cells had a basal domain that is approximately 2 μm taller than adjacent low ankyrin cells. Actin and ankyrin-costained specimens also showed that the prominent ankyrin ring is basal to the circumferential actin bundle (not shown).

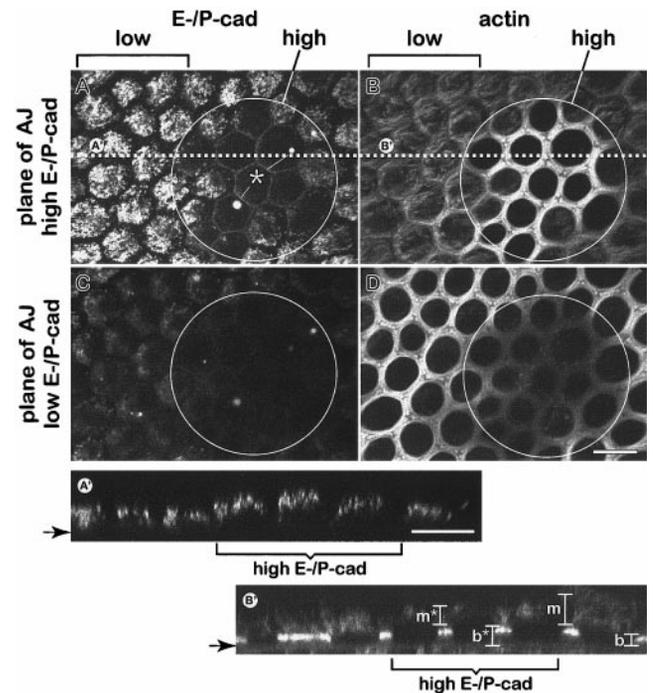


FIGURE 6. Confocal images of a group of high E-/P-cadherin (E-/P-cad) cells surrounded by low E-/P-cadherin cells, dually stained for the cadherins and for actin. All images are of the same field but show scans at different planes. Scans at the plane of the adherens junction (AJ), where cadherins codistribute with the actin circumferential microfilament bundle, are shown for both the high E-/P-cadherin (A and B, scans 52–54) and low E-/P-cadherin (C and D, scans 61–63) portions of the field. Scan numbering (0.2 μm scans) is based on actin staining, where scan 1 is the apex of the microvilli of the tallest cell and scan 75 is the cell base. Junctional staining for E-/P-cadherin is seen at the plane of the AJ for high (A) but not low (C) E-/P-cadherin cells. An asterisk indicates lipofuscin granules at the plane of the AJ (A). Comparative heights of microvillar and cell body domains in low and high E-/P-cadherin cells are shown in a cross-sectional Z-scan of actin (B'); the position of the Z-scan is indicated by dotted lines in en face actin image (B). In the actin Z-scan (B'), the heights of the cell body (b) and apical microvillar (m) domains are indicated for high E-/P-cadherin cells (b*, m*) and flanking low cells (b, m). The AJ in the high E-/P-cadherin cells is at the plane where autofluorescent granules are located (base of microvilli) in adjacent low E-/P-cadherin cells (A). A Z-scan of autofluorescent granules (A') illustrates the differing heights of the cell bodies in low and high E-/P-cadherin fields. Scale bars, 20 μm . Scale bar in (D) is for all composite images; scale bar in (A') is for the Z-scans.

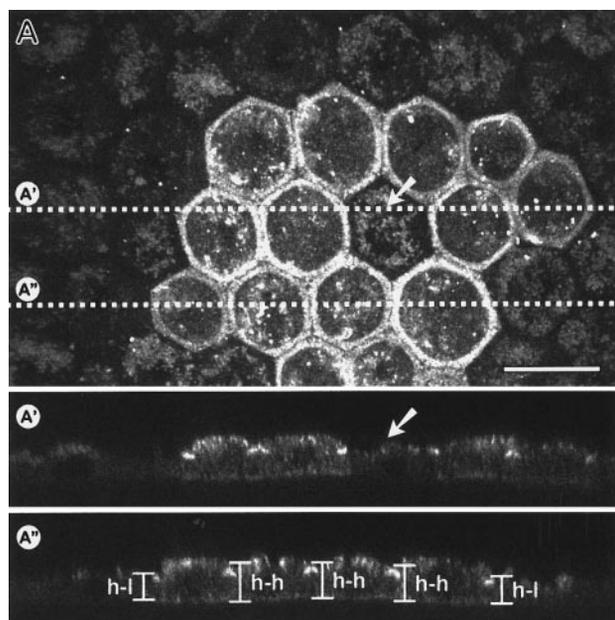


FIGURE 7. Composite confocal image (A) and two Z-scans (A', A'') of a high E/P-cadherin region immunostained for ankyrin showing a cluster of high ankyrin cells. (Cadherin staining is not shown.) The positions of Z-scans A' and A'' are indicated by dotted lines A' and A'', respectively, on the composite image. In Z-scan (A'), a single shorter low ankyrin cell (arrow in A and A') is flanked by taller high ankyrin cells. Z-scan (A'') illustrates the height of the cell body domain, from the peripheral ankyrin ring to the cell base. Cell height is greater at the borders of two high ankyrin cells (brackets *b-b*) than at the borders of adjacent high and low ankyrin cells (brackets *b-l*). Scale bar, 20 μm .

RPE Cells with High Ankyrin: Codistribution with Na/K ATPase

Within fields of RPE cells with high E/P-cadherin, individual cells may have high or low ankyrin (Figs. 3D, 4B), and high or low Na/K ATPase (Figs. 3F, 4C), but ankyrin and Na/K ATPase are not necessarily high in the same cells. Fields were selected that contained cells with all combinations of staining patterns to examine the subcellular codistribution of the two proteins (Fig. 8). Ankyrin staining was seen in apical microvilli, although the staining was not prominent and was not greater in cells with high levels of Na/K ATPase (Figs. 8B, 8G). Ankyrin was enriched in a plane at the base of microvilli, which has been called a terminal web-like region,¹⁹ where it codistributed with Na/K ATPase (Figs. 8C, 8H). Na/K ATPase did not codistribute with the prominent peripheral rings of ankyrin (Figs. 8D, 8I, cell 2). However, Na/K ATPase and ankyrin distinctly colocalized in the cell bases; even cells with low overall levels of Na/K ATPase but high ankyrin showed a basal codistribution of the sodium pump with ankyrin (Figs. 8E, 8J).

The differences that were observed in cell height and Na/K ATPase polarity between bovine RPE cells with low and those with high E/P-cadherin levels are summarized in the diagram shown in Figure 9.

DISCUSSION

One general observation resulting from examining protein distributions in individual RPE cells in situ was that protein ex-

pression levels among cells are distinctly heterogeneous. All proteins examined in the present study, with the exception of F-actin, showed this heterogeneity. RPE cells appear uniform in phenotype due to their regular hexagonal organization, but this gross uniformity gives a misimpression of the molecular heterogeneity of the cells. Differences among RPE cells in the expression levels of the intermediate filament protein vimentin were previously shown,^{20,21} and amounts of melanin also vary among cells.²² Because large expanses of the monolayer were examined here, we found that the cell-cell variations in expression for different proteins occurred in different patterns. Cells with high expression levels for a given protein could occur predominantly as single cells (Na/K ATPase), small clusters or rows of cells (ankyrin), larger groups of cells (fodrin), or more expansive fields of cells (E/P-cadherin). The monolayer is, therefore, a mosaic, with individual cells having the potential to exhibit a complicated spectrum of protein expression levels. We previously hypothesized that RPE cells are phenotypically heterogeneous in culture because of a naturally occurring heterogeneity in expression levels of proteins that affect cell shape.^{21,23} One such protein is E-cadherin, which has a morphoregulatory function,^{11,24-26} and which was found in the present study to vary among cells. This observation, coupled with the observation that E-cadherin is expressed by some but not all phenotypes of cultured RPE cells,⁷ lends support to this hypothesis.

The protein with the most striking intercellular variability was Na/K ATPase, which was found at markedly high levels in a subset of RPE cells. These cells varied in frequency among eyes but were consistently less numerous in the posterior pole. This unexpected observation helps explain our previous observation that the number of sodium pump sites, quantified by ouabain binding, varies among eyes and is lower in the bovine area centralis relative to the peripheral retina.²⁷ Similar results were obtained when macular and extramacular regions of human eyes were compared.²⁷ At the time we assumed that the entire population of RPE cells in the macula/area centralis had an overall lower pump site density than more peripheral cells. It appears now that lower macular pump density is due to a lower frequency of cells with high Na/K ATPase expression levels. The functional ramifications of this observation remain obscure. Ion transport for the entire tissue may be similar whether all cells have the same number of pumps or individual cells have varying numbers of pumps, provided that the total number of sodium pump sites is adequate for the tissue surface area. However, it seems inevitable that large intercellular differences in sodium pump density will impact ion transport in the microenvironment of individual RPE cells and the photoreceptors with which they interact.

RPE cells have been known for many years to have a predominantly apical distribution of Na/K ATPase within eyes.¹²⁻¹⁸ This polarity was confirmed in the present study in that Na/K ATPase immunostaining was clearly seen on the apical microvilli of bovine RPE cells. However, closer examination revealed heterogeneity in the distribution of the sodium pump among cells. Certain subcellular distributional patterns of Na/K ATPase could be predicted by levels of E/P-cadherin coexpression in the same cells. Compared with RPE cells with low E/P-cadherin levels, cells with high levels showed reduced apical polarization of Na/K ATPase, especially if the cells also expressed high levels of ankyrin. The reduced apical polarity of Na/K ATPase in RPE cells with high E/P-cadherin level resulted

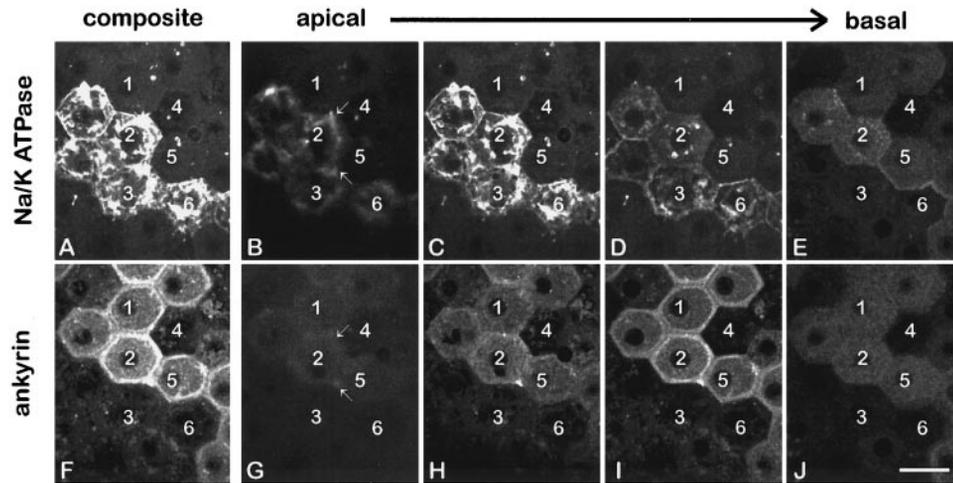


FIGURE 8. Confocal images of RPE cells dual-stained for Na/K ATPase (A through E) and ankyrin (F through J, respectively). The first pair of images (A and F) are composites of all scans. Six cells are identified by numbers. These include cells with high staining for both proteins (*cell 2*), high Na/K ATPase and low ankyrin (*cells 3 and 6*), low Na/K ATPase and high ankyrin (*cells 1 and 5*), or low staining for both (*cell 4*). The remaining pairs of images are each composites of three scans (0.6 μm total) at different planes along the apical-to-basal axis: (B and G; scans 6–8), apex of the apical microvilli; (C and H; scans 15–17), base of the microvillar region (terminal web-like zone); (D and I; scans 20–22), plane of the prominent peripheral ankyrin ring in cell 2; (E and J; scans 30 to 32), basal membrane domain. Scan number is based on Na/K ATPase staining where scan 1 is the apex of the tallest cell and scan 40 is the cell base. Ankyrin staining was not so prominent in microvilli as elsewhere in the cell, although codistribution with Na/K ATPase in microvilli can be detected in cells with high levels of both proteins (*cell 2*, arrows in B and G). In the region of the peripheral ankyrin ring (*cell 2* in D), pericellular Na/K ATPase is diminished (*cell 2* in D). (All cells in the field are not of identical height so the plane of this pair of scans was selected to show the zonular ring of ankyrin in the cell [*cell 2*] which is highly stained for both ankyrin and Na/K ATPase.) At the basal membrane, Na/K ATPase and ankyrin codistribute (E and J). In this domain, cells that showed low Na/K ATPase in other subcellular domains showed distinct basal staining only in cells with high ankyrin (see *cells 1 and 5*). At the basal surface, the pattern of cells with distinct staining for both ankyrin and Na/K ATPase appears to be the same. Scale bar, 20 μm .

from a combination of a smaller microvillar domain, a taller lateral domain, and increased immunodetectable Na/K ATPase in basolateral membranes (see diagram in Fig. 9). E-cadherin has been shown to induce a basal polarity of Na/K ATPase in epithelial cells in vitro,^{10,11} including in an RPE cell line.⁴ The increased basal distribution of Na/K ATPase in RPE cells in situ with higher levels of E-/P-cadherin is consistent with the “basal” inductive properties of E-cadherin.

One of the mechanisms for polarizing Na/K ATPase to basolateral membranes of epithelial cell lines is selective retention via ankyrin linkage to the basal fodrin-actin membrane cytoskeleton.²⁸ Ankyrin and fodrin were reported to show a parallel reversal with Na/K ATPase to the apical surface of rat RPE cells,¹⁸ suggesting that an ankyrin-fodrin-selective retention mechanism may contribute to polarizing the pump to apical membranes in the RPE. There is a question, however, about the role played by at least fodrin in contributing to apical pump polarity. During later stages of chick embryogenesis as microvilli elongate, fodrin (spectrin) no longer codistributes with Na/K ATPase.¹⁹ Rather, fodrin is enriched at the base of microvilli (terminal web-like region) and in the base of cells.¹⁹ In our analysis of bovine RPE cells in situ shown here, ankyrin and fodrin were detected in apical microvilli, but neither protein distributed prominently to this region. Of note, cells with high levels of Na/K ATPase did not necessarily show comparably elevated levels of ankyrin and fodrin, and distinct codistribution with apical Na/K ATPase was difficult to find, even in

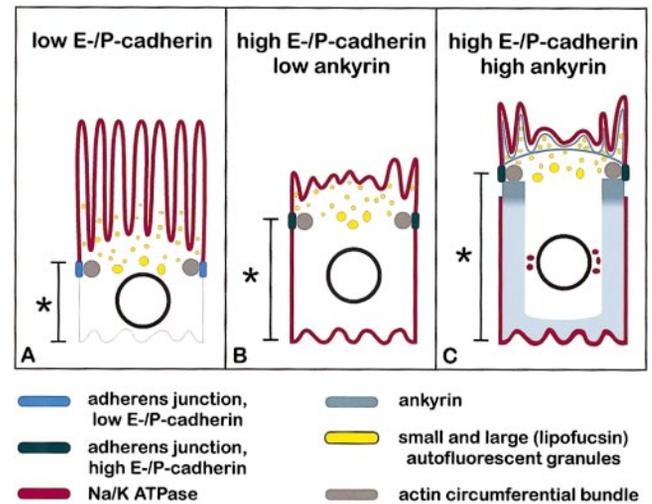


FIGURE 9. Diagram summarizing the protein distributions in low E-/P-cadherin RPE cells (A) and in high E-/P-cadherin cells with either low (B) or high (C) ankyrin levels. In (A), Na/K ATPase polarity is predominantly apical due to a large microvillar domain where Na/K ATPase distributed and a short lateral domain of the cell body (*) with no detectable Na/K ATPase. As compared with cells in (A), cells in (B) have a reduced apical polarity of Na/K ATPase due to a reduced size of the microvillar domain and an increased lateral domain (*) where Na/K ATPase was detected. Cells in (C) have an even less apically polarized Na/K ATPase due to a greater enlargement of the lateral domain (*) and an enrichment of Na/K ATPase in basolateral membranes.

cells with high levels of both Na/K ATPase and ankyrin or fodrin. The lack of prominent codistribution between Na/K ATPase and ankyrin-fodrin in apical microvilli raises the possibility that another linking system contributes to stabilizing apical Na/K ATPase in bovine RPE cells, as has been previously suggested for chick RPE.¹⁹

The ankyrin-fodrin cytoskeleton may play a greater role in segregating Na/K ATPase to the basal membranes of RPE in situ, particularly in high E/P-cadherin cells. Only in high E/P-cadherin cells were high levels of ankyrin and fodrin seen, and both proteins distributed more prominently basal to the adherens junction where a specific codistribution of at least ankyrin with Na/K ATPase was seen. E-cadherin expression may change the expression of fodrin and ankyrin and induce their basolateral enrichment, thereby contributing to a basolateral enrichment of Na/K ATPase. Support for this possibility comes from studies of cultured RPE cells transfected to express E-cadherin.⁴ Transfected cells showed increased amounts of fodrin and a change in ankyrin isoform accompanying the development of a basal Na/K ATPase. Whether the higher levels of ankyrin seen here in some high E/P-cadherin RPE cells reflect a different ankyrin isoform is currently unknown because the ankyrin antibodies that were used recognize all isoforms.

We focused in the present study on ankyrin-fodrin-cytoskeletal linkage as a mechanism for polarizing Na/K ATPase, because regulating the expression of membrane cytoskeletal proteins and their position within cells are functions that have been attributed to E-cadherin. However, there are several mechanisms for polarizing Na/K ATPase that are differentially used by different epithelial tissues,²⁹ and it is unlikely that mechanisms triggered by E-cadherin are acting alone in RPE cells. Indeed, RPE cells in situ with high E/P-cadherin levels show an increased basal polarity of Na/K ATPase, but the cells nonetheless retain distinct apical Na/K ATPase. If the increased basal distribution is induced by E/P-cadherin, then it appears that cadherin-mediated mechanisms compete, not entirely successfully, with other mechanisms in RPE cells that maintain an apical sodium pump. What the latter mechanisms are, and why E/P-cadherin is only partially effective, remain to be determined.

Acknowledgment

The authors thank Emmer Foods, Inc. for generously providing cow eyes.

References

- Gundersen D, Powell SK, Rodriguez-Boulan E. Apical polarization of N-CAM in retinal pigment epithelium is dependent on contact with the neural retina. *J Cell Biol.* 1993;121:335-343.
- Davis AA, Bernstein PS, Bok D, Turner J, Nachtigal M, Hunt RC. A human retinal pigment epithelial cell line that retains epithelial characteristics after prolonged culture. *Invest Ophthalmol Vis Sci.* 1995;36:955-964.
- Huotari V, Sormunen R, Lehto V-P, Eskelinen S. The polarity of the membrane skeleton in retinal pigment epithelial cells of developing chicken embryos and in primary culture. *Differentiation.* 1995;58:205-215.
- Marrs JA, Andersson-Fisone C, Jeong MC, et al. Plasticity in epithelial cell phenotype: modulation by expression of different cadherin cell adhesion molecules. *J Cell Biol.* 1995;129:507-519.
- Grunwald GB. Cadherin cell adhesion molecules in retinal development and pathology. *Prog Retin Eye Res.* 1996;15:363-392.
- McKay BS, Irving PE, Skumatz CMB, Burke JM. Cell-cell adhesion molecules and the development of an epithelial phenotype in cultured human retinal pigment epithelial cells. *Exp Eye Res.* 1997;65:661-671.
- Burke JM, Cao F, Irving PE, Skumatz CMB. Expression of E-cadherin by human retinal pigment epithelium: delayed expression in vitro. *Invest Ophthalmol Vis Sci.* 1999;40:2963-2970.
- Redies C, Muller HA. Similarities in structure and expression between mouse P-cadherin, chicken B-cadherin and frog XU/B-cadherin. *Cell Adhes Commun.* 1994;2:511-520.
- Murphy-Erdosh C, Napolitano EW, Reichardt LF. The expression of B-cadherin during embryonic chick development. *Dev Biol.* 1994;161:107-125.
- Nelson WJ, Shore EM, Wang AZ, Hammerton RW. Identification of a membrane-cytoskeletal complex containing the cell adhesion molecule uvomorulin (E-cadherin), ankyrin, and fodrin in Madin-Darby Canine kidney epithelial cells. *J Cell Biol.* 1990;110:349-357.
- Nelson WJ. Regulation of cell surface polarity from bacteria to mammals. *Science.* 1992;258:948-955.
- Steinberg RM, Miller SS. Transport and membrane properties of the retinal pigment epithelium. In: Zinn KM, Marmor MF, eds. *The Retinal Pigment Epithelium.* Cambridge: Harvard University Press; 1979:205-225.
- Bok D. Autoradiographic studies on the polarity of plasma membrane receptors in RPE cells. In: Hollyfield J, ed. *The Structure of the Eye.* New York: Elsevier North Holland; 1982:247-256.
- Bok D. The retinal pigment epithelium, a versatile partner in vision. *J Cell Sci Suppl.* 1993;17:189-195.
- Caldwell RB, McLaughlin BJ. Redistribution of Na-K-ATPase in the dystrophic rat retinal pigment epithelium. *J Neurocytol.* 1984;13:895-910.
- Okami T, Yamamoto A, Omori K, Takada T, Uyama M, Tashiro Y. Immunocytochemical localization of Na,K-ATPase in rat retinal pigment epithelial cells. *J Histochem Cytochem.* 1990;38:1267-1275.
- Rizzolo LJ. The distribution of Na/K ATPase in the retinal pigmented epithelium from chicken embryo is polarized in vivo but not in primary cell culture. *Exp Eye Res.* 1990;51:435-446.
- Gundersen D, Orłowski J, Rodriguez-Boulan E. Apical polarity of Na,K-ATPase in retinal pigment epithelium is linked to a reversal of the ankyrin-fodrin submembrane cytoskeleton. *J Cell Biol.* 1991;112:863-872.
- Rizzolo LJ, Zhou S. The distribution of Na⁺,K⁺-ATPase and 5A11 antigen in apical microvilli of the retinal pigment epithelium is unrelated to alpha-spectrin. *J Cell Sci.* 1995;108:3623-3633.
- Owaribe K, Kartenbeck J, Rungger-Brandle E, Franke WW. Cytoskeletons of retinal pigment epithelial cells: interspecies differences of expression patterns indicate independence of cell function from the specific complement of cytoskeletal proteins. *Cell Tissue Res.* 1988;254:301-315.
- Burke JM, Skumatz CMB, Irving PE, McKay BS. Phenotypic heterogeneity of retinal pigment epithelial cells in vitro and in situ. *Exp Eye Res.* 1996;62:63-73.
- Pollack JS, Kim JE, Pulido JS, Burke JM. Tissue effects of subclinical diode laser treatment of the retina. *Arch Ophthalmol.* 1998;116:1633-1639.
- Burke JM. Determinants of RPE phenotype and polarity. In: Marmor MF, Wolfensberger TJ, eds. *Retinal Pigment Epithelium—Current Aspects of Function and Disease.* London: Oxford University Press; 1998:86-102.
- Mays RW, Nelson WJ, Marrs JA. Generation of epithelial cell polarity: roles for protein trafficking, membrane-cytoskeleton, and E-cadherin-mediated cell adhesion. *Cold Spring Harb Symp Quant Biol.* 1995;60:763-773.
- Takeichi M. Morphogenetic roles of classic cadherins. *Curr Opin Cell Biol.* 1995;7:619-627.
- Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 1996;84:345-357.
- Burke JM, McKay BS, Jaffe GJ. Retinal pigment epithelial cells of the posterior pole have fewer Na/K adenosine triphosphatase pumps than peripheral cells. *Invest Ophthalmol Vis Sci.* 1991;32:2042-2046.
- Hammerton RW, Krzeminski KA, Mays RW, Ryan TA, Wollner DA, Nelson WJ. Mechanism for regulating cell surface distribution of Na⁺,K⁺-ATPase in polarized epithelial cells. *Science.* 1991;254:847-850.
- Rizzolo LJ. Polarization of the Na⁺,K⁺-ATPase in epithelia derived from the neuroepithelium. *Int Rev Cytol.* 1999;185:195-235.