

Membrane Polarity of the Na⁺–K⁺ Pump in Primary Cultures of *Xenopus* Retinal Pigment Epithelium

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The retinal pigment epithelium is a transporting epithelium that helps regulate the volume and composition of the subretinal space surrounding photoreceptor outer segments. The capacity of the RPE to actively transport Na⁺ and K⁺ between the retina and the blood supply depends on the localization of the Na⁺, K⁺-ATPase to the apical membrane, but in culture this polar distribution can be lost. Using primary cultures of *Xenopus* RPE, we examined the anatomical and functional polarity of this electrogenic pump. Confluent monolayers were established on Matrigel-coated microporous filters and cultured for 2–4 weeks in serum-free defined medium. Electrogenic pump activity at the apical and basolateral membranes was assayed by mounting the monolayer and filter in an Ussing chamber and exposing one or the other surface to ouabain while recording the apical (V_{ap}) and basolateral (V_{ba}) membrane potentials with an intracellular microelectrode. The addition of 0.2 mM ouabain to the apical bath caused V_{ap} to rapidly depolarize by about 4 mV, consistent with the inhibition of a hyperpolarizing pump current at that membrane. When ouabain was added to the basal bath, however, it had no effect on V_{ba} , suggesting the absence of a functional Na⁺-K⁺ pump on the basolateral membrane. To confirm these electrophysiological results, we examined the distribution of the Na⁺, K⁺-ATPase catalytic component using an antiserum specific for the bovine kidney α subunit. Antibody labeling of cultures was highly polarized, with strong reaction present on the apical microvilli, but not the basolateral cell surfaces. The findings of this study indicate that the Na⁺-K⁺ pump in monolayers of *Xenopus* RPE, as in native RPE, is located mainly in the apical membrane, providing evidence of a functionally intact transport pathway in these primary cultures.

Key words: cell culture; cell polarity; confocal microscopy; defined medium; frog; immunocytochemistry; Na⁺, K⁺-ATPase; ouabain; retinal pigment epithelium; transepithelial resistance.

1. Introduction

The retinal pigment epithelium (RPE) is a simple cuboidal epithelium that has a close anatomical and functional relationship with the photoreceptors in the distal retina. Like other epithelia, RPE cells are highly polarized, with distinct apical and basolateral membrane domains that differ from one another both morphologically and functionally. One of the distinguishing features of the RPE is the apical location of the Na⁺-K⁺ pump (Bok, 1982; Gunderson, Orłowski and Rodriguez-Boulan, 1991; Miller, Steinberg and Oakley, 1978; Ostwald and Steinberg, 1980). In all other epithelia, with the exception of that of the choroid plexus (Quinton, Wright and Tormey, 1973; Siegel et al., 1984), the Na⁺-K⁺ pump is restricted to the basolateral membrane (Ernst and Hootman, 1981). The functional significance of the apical location of the Na⁺-K⁺ pump is that it enables the RPE to actively secrete Na⁺ into, and absorb K⁺ out of, the subretinal space (Miller and Steinberg, 1977).

Cultured RPE from a variety of species have been utilized as model systems for the study of a number of

RPE functions, including signal transduction (Frambach et al., 1990), ion transport (Kennedy, 1992), retinal attachment (Defoe and Easterling, 1994) and outer segment phagocytosis (Edwards and Szamier, 1977; Chaitin and Hall, 1983). A potential limitation of these *in vitro* systems is that the properties of cultured cells may differ from those of the native epithelium. For example, recent reports have described changes in the types of ion channels expressed (Wen, Lui and Steinberg, 1993), as well as changes in the distribution of Na⁺, K⁺-ATPase (Rizzolo, 1990), in RPE cells from confluent monolayers that possess a highly differentiated morphology. The reasons for these *in vitro* changes are not understood, but the type of substrate, composition of the culture medium and number of passages are possible factors.

In this study, we used electrophysiological and immunocytochemical techniques to investigate the membrane distribution of Na⁺, K⁺-ATPase in primary cultures of *Xenopus* RPE grown on Matrigel-coated polycarbonate filters in a defined medium. Parts of this study have been published in preliminary form (Defoe et al., 1994).

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2. Materials and Methods

Cell Culture

Animals African clawed frogs (post-metamorphic) were obtained from *Xenopus I* (Ann Arbor, MI, U.S.A.) and maintained at 24–26°C on a daily lighting schedule of 12 hr dark:12 hr light. Animals were killed by decapitation and, prior to removal of eyes, both the brain and spinal cord were pithed.

Culture medium For reconstruction of cell monolayers in culture, a defined medium was used which consisted of the basal medium NCTC-135, diluted to 75% strength with HPLC-grade water and supplemented with the following nutrients and hormones (at the indicated final concentration): insulin (10 µg ml⁻¹), transferrin (5.5 µg ml⁻¹), sodium selenite (6.7 ng ml⁻¹), sodium pyruvate (0.11 mg ml⁻¹) (GMS-A supplement), linoleic acid-albumin (10 µg ml⁻¹ linoleic acid, 1 mg ml⁻¹ BSA), hydrocortisone (20 nM), triiodothyronine (10 nM), putrescine (0.3 µg ml⁻¹), aprotinin (50 µg ml⁻¹), basic fibroblast growth factor (human recombinant; 0.1 ng ml⁻¹), epidermal growth factor (murine; 50 ng ml⁻¹), penicillin-streptomycin solution (100 U ml⁻¹ and 0.1 mg ml⁻¹, respectively) and Fungizone (0.25 µg ml⁻¹ amphotericin B). All reagents were purchased from Gibco (Grand Island, NY, U.S.A.), with the exception of linoleic acid-albumin, hydrocortisone, triiodothyronine, putrescine and aprotinin, which were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Preparation of RPE monolayers Primary cultures of *Xenopus laevis* pigment epithelium were established as described previously (Defoe and Easterling, 1994). Briefly, sheets of RPE were harvested from eyes treated with the neutral protease dispase (Grade II; Boehringer Mannheim, Indianapolis, IN, U.S.A.). Following trypsinization, individual cells and cell clusters were plated at a density of 1.5×10^5 – 3×10^5 cells cm⁻² on microporous membrane filters (Transwells; 6.5-mm diameter, 0.4-µm pore size; Costar, Cambridge, MA, U.S.A.) coated with Matrigel (Collaborative Biomedical Products, Bedford, MA, U.S.A.). Approximately 150 µl of defined medium was maintained in the upper chamber of the Transwells and 700 µl in the multiwell plate (lower chamber). Cultures were placed in a modular incubation chamber (Billups-Rothenberg, Del Mar, CA, U.S.A.) which was gassed with a humidified 10% O₂: 3% CO₂: 87% N₂ mixture and maintained at 26°C in an environmental incubator. One-half of the medium volume in each Transwell chamber was replaced three times weekly with freshly prepared medium. To monitor reconstruction of RPE monolayers, resistance measurements were performed on individual cultures using an epithelial voltammeter (EVOM; WPI, Sarasota, FL, U.S.A.). All of the experiments reported made use of cultures which had achieved confluence, as judged by the attainment of a maximum transepithelial resistance.

Immunological Procedures

Immunofluorescence microscopy Freshly dissected epithelium-choroid-sclera (free of neural retina) and kidney, as well as filter-grown RPE cells, were fixed for 2 hr at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After several washes in phosphate-buffered saline, pH 7.4 (PBS), the polycarbonate membranes were cut out of their polystyrene mounts. Specimens were then processed directly for immunocytochemistry (see below), or infused with 20% sucrose in PBS and frozen in liquid Freon 22 prior to obtaining 10-µm sections with a cryostat. For cell labeling studies, we used an antiserum directed against electrophoretically purified bovine Na⁺, K⁺-ATPase α subunit (31B; gift of Dr Stephen Ernst), which as been extensively characterized using bovine and mouse tissues (Hieber et al., 1989; Mata et al., 1991). This reagent was diluted 1:300 in Tris-buffered saline, pH 7.4 (TBS) + 1% bovine serum albumin (BSA) and 0.1% Triton X-100, and incubated with tissue sections or monolayer flat-mounts overnight at 4°C. After several washes in TBS-BSA, antibody was detected using a 1:40 dilution of goat anti-rabbit Ig coupled to Texas Red (incubation for 1 hr at room temperature; Southern Biotechnology Associates, Birmingham, AL, U.S.A.). Specimens were viewed using an Axiophot microscope equipped with a 20X neofluor objective and epifluorescence optics (Carl Zeiss, Thornwood, NY, U.S.A.). Photographs were taken using Tmax 400 film (Eastman Kodak, Rochester, NY, U.S.A.).

For confocal microscopy, immunostained eyecup sections, or pieces of polycarbonate membrane with attached RPE cells, were imaged using a Zeiss Axiovert microscope equipped with a 63X oil immersion lens (Carl Zeiss) and interfaced to an MRC-600 (BioRad Microscience Division, Cambridge, MA, U.S.A.). Serial optical sections were collected at approximately 0.5-µm interval steps in the z-axis and stored on 940 MB optical disk cartridges. Confocal images were then selected and, after conversion to the appropriate file format, transferred to Tmax 100 film (Eastman Kodak) using a Polaroid Palette (Polaroid, Cambridge, MA, U.S.A.).

Western Blotting RPE sheets obtained from Dis-pase-treated eyes were washed extensively in amphibian balanced salt solution (Defoe and Easterling, 1994), then homogenized in 50 mM Tris-HCl, pH 7.4 containing 2% Nonidet P-40, 120 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂ and protease inhibitors [0.7 µg ml⁻¹ pepstatin, 0.5 µg ml⁻¹ leupeptin, and 1 mM Pefabloc (Boehringer-Mannheim)] using an ultrasonic cell disruptor (Kontes, Vineland, NJ, U.S.A.). After centrifugation at 17 530 g for 15 min at 4°C, the supernatant was removed and stored at -70°C. Soluble tissue extracts, or biotinylated molecular weight markers (broad range; BioRad), were mixed 1:1 (v/v) with sample buffer containing 4% SDS and

2.5% β -mercaptoethanol. Samples were electrophoresed on 7.5% denaturing polyacrylamide gels in a Mini-protein II apparatus (BioRad) and the separated proteins transferred to nitrocellulose. After being blocked overnight at 4°C in TBS containing 10% non-fat dry milk, individual lanes of the protein blot were incubated with rabbit anti-bovine Na^+ , K^+ -ATPase α subunit (1:6000 dilution in TBS-BSA), then with HRP-conjugated goat anti-rabbit Ig (1:1000 dilution in TBS-BSA; Southern Biotechnology Associates). Enzyme reaction was developed using the enhanced chemiluminescence (ECL) system (Amersham Life Sciences, Arlington, IL, U.S.A.). To assess possible non-specific interactions, primary antibody was either omitted or replaced by a control rabbit IgG ($5 \mu\text{g ml}^{-1}$; Vector Laboratories, Burlingame, CA, U.S.A.). Molecular weight markers were visualized after transfer to nitrocellulose using avidin-HRP (BioRad) in combination with ECL detection.

Electrophysiology

Solutions In electrophysiological experiments, monolayers were superfused with an amphibian Ringers solution consisting of 110 mM NaCl, 2 mM KCl, 5 mM HEPES-NaOH, 1.8 mM CaCl_2 , 1 mM MgCl_2 and 10 mM glucose. This solution had a pH of 7.4 when equilibrated with room air. The concentration of K^+ was varied by replacing NaCl with KCl. The osmolality of the external solution was $230 \pm 5 \text{ mosmol kg}^{-1}$.

Recording chamber Polycarbonate membranes supporting confluent RPE monolayers were excised from their polystyrene mounts, placed apical-side up on a nylon mesh, and then sandwiched between the two halves of a modified Ussing chamber (Joseph and Miller, 1991). The upper half of the chamber contained two concentric ridges that formed a mechanical seal on the apical surface of the RPE, exposing a surface area of 0.07 cm^2 . The apical and basolateral surfaces of the RPE were superfused independently by gravity feed at a rate of approximately 10 chamber volumes min^{-1} . Solution composition changes were made at a manifold that was separated from the chamber by a dead space of approximately 1 ml.

Recording configuration The techniques used for electrophysiological recordings were similar to those used in previous studies (Fujii et al., 1992). In both the apical and basal compartments of the recording chamber, a calomel electrode made electrical contact with the perfusion solution via a 150 mM KCl-agar bridge. The transepithelial potential (TEP) (apical-side positive) was recorded differentially between the two calomel electrodes. Conventional microelectrodes were pulled from borosilicate tubing on a horizontal puller (Sutter Instruments, San Rafael, CA, U.S.A.). When filled with 150 mM KCl, microelectrodes had a tip resistance of 150 to 250 M Ω . Membrane potentials were measured by impaling a cell in the monolayer

across the apical membrane with a microelectrode connected to a high impedance amplifier (Axoprobe 1A, Axon Instruments, San Rafael, CA, U.S.A.). All voltage signals were digitized and stored on a microcomputer for subsequent analysis.

The apical membrane potential (V_{ap}) was determined by referencing the intracellular microelectrode to the calomel electrode in the apical bath; the basal membrane potential (V_{ba}) was calculated from the relation, $\text{TEP} = V_{\text{ba}} - V_{\text{ap}}$. The transepithelial resistance (R_t) and the apparent ratio of the apical to basolateral membrane resistance $R_{\text{ap}}/R_{\text{ba}}$ (a value) were obtained by passing $2 \mu\text{A}$ current pulses across the tissue and monitoring the appropriate voltage responses. The resistance of the Transwell filter devoid of RPE cells was approximately $10 \Omega \text{ cm}^2$ and was ignored in calculating R_t and $R_{\text{ap}}/R_{\text{ba}}$. The transepithelial resistance is given by

$$R_t = \frac{R_s(R_{\text{ap}} + R_{\text{ba}})}{R_{\text{ap}} + R_{\text{ba}} + R_s}$$

where R_s is the combined resistance of the paracellular pathway and the edge damage caused by the mechanical seal at the margins of the tissue.

It has been shown previously that the electrogenic $\text{Na}^+\text{-K}^+$ pump in the apical membrane generates a source of steady current, I_{pump} (Miller et al., 1978). Part of this current flows through R_{ap} , hyperpolarizing V_{ap} , and the remainder flows through R_s and R_{ba} , hyperpolarizing V_{ba} . The hyperpolarizations of V_{ap} and V_{ba} due to the $\text{Na}^+\text{-K}^+$ pump current are given by

$$\Delta V_{\text{ap}} = i_{\text{pump}} \left[\frac{R_{\text{ap}}(R_{\text{ba}} + R_s)}{R_{\text{ap}} + R_{\text{ba}} + R_s} \right] \quad (1)$$

$$\Delta V_{\text{ba}} = i_{\text{pump}} \left[\frac{(R_{\text{ap}})(R_{\text{ba}})}{R_{\text{ap}} + R_{\text{ba}} + R_s} \right] \quad (2)$$

If R_s is small relative to R_{ba} , then changes in pump current will alter V_{ap} and V_{ba} by the same amount.

3. Results

Electrophysiology

Electrical parameters under control conditions The transepithelial resistance (R_t) of *Xenopus* monolayers mounted in the chamber averaged $102 \pm 39 \Omega \text{ cm}^2$ ($n = 12$; range: $40\text{--}175 \Omega \text{ cm}^2$). The lower values probably result from edge damage produced by the mechanical seal. In four monolayers, transepithelial resistances greater than $150 \Omega \text{ cm}^2$ were achieved by coating the sealing ridges with a thin layer of Sylgard elastomer (Dow Corning, Midland, MI, U.S.A.), which apparently minimized edge damage. These values approach those measured across the monolayers prior to excision from the Transwell inserts ($244 \pm 30 \Omega \text{ cm}^2$).

Immediately after mounting the monolayer in the recording chamber, the TEP transiently increased

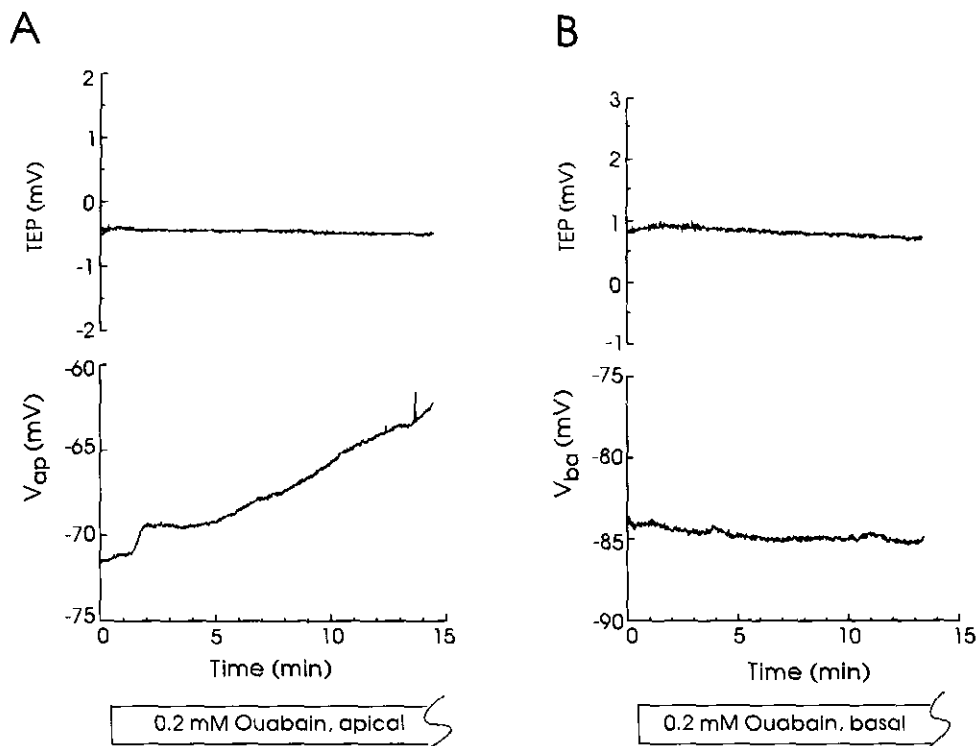


FIG. 1. Effects of apical and basal ouabain on membrane voltage. (A) 0.2 mM ouabain was superfused through the apical compartment of the recording chamber at the time indicated by the open bar. This depolarized the apical membrane potential (V_{ap}) in two phases, a fast phase attributable to the inhibition of the electrogenic $\text{Na}^+\text{-K}^+$ pump and a slow phase presumably due to the run down of K^+ and Na^+ gradients across the cell membranes. The transepithelial potential did not change, indicating that the basolateral membrane potential followed the changes in V_{ap} . (B) 0.2 mM ouabain was superfused through the basal compartment of the chamber holding a different RPE monolayer during the time indicated. Basal ouabain had no effect on either V_{ap} or TEP, suggesting the absence of an electrogenic $\text{Na}^+\text{-K}^+$ pump at the basolateral membrane.

from about 2 to 5 mV, and then decreased to a steady state near zero. In 12 monolayers, the steady-state TEP averaged 0.7 ± 0.8 mV. Intracellular microelectrode recordings that were stable for 5 min or more were considered acceptable for analysis. In 11 cells in as many monolayers, the apical membrane potential (V_{ap}) averaged -75.6 ± 6.7 mV (range: -86 to -65 mV). The basal membrane potential (V_{ba}) in these cells was not significantly different from V_{ap} , as expected from the near zero TEP values. The ratio of apical to basolateral membrane resistances (R_{ap}/R_{ba}) averaged 0.34 ± 0.23 (range: 0.1 to 0.8).

Effects of apical and basal ouabain We used ouabain to probe the apical and basolateral membranes of cultured *Xenopus* RPE for the activity of the electrogenic $\text{Na}^+\text{-K}^+$ pump. It has been demonstrated in the bullfrog RPE that the electrogenic $\text{Na}^+\text{-K}^+$ pump generates an outward current that hyperpolarizes the apical and basolateral membranes and that poisoning the $\text{Na}^+\text{-K}^+$ pump with ouabain causes a rapid depolarization of V_{ap} due to inhibition of the pump current (Miller et al., 1978). Following this, V_{ap} depolarizes more slowly as the transmembrane Na^+ and K^+ gradients dissipate.

Figure 1(A) shows the effect of apical ouabain on V_{ap} and TEP in a *Xenopus* RPE monolayer. At the start of the experiment, both surfaces of the monolayer were superfused with normal Ringers solution and

V_{ap} was -72 mV. At $t = 30$ sec, the apical superfusate was switched from normal Ringers to Ringers containing 0.2 mM ouabain. After a 60-sec delay that was due to the clearance of dead space and diffusion of ouabain through the unstirred layer outside the apical membrane (see below), V_{ap} underwent a biphasic depolarization. In the first phase, V_{ap} rapidly depolarized by approximately 4 mV. Following this, V_{ap} briefly stabilized and then depolarized at a slower rate. During the rapid depolarization of V_{ap} , which presumably represents the inhibition of the $\text{Na}^+\text{-K}^+$ pump current, V_{ba} followed closely (not shown) and TEP remained virtually unchanged (upper panel). For five monolayers, the depolarization of V_{ap} during the fast phase averaged 3.8 ± 1.2 mV.

In contrast to the response of V_{ap} when ouabain was added to the apical superfusate, basal ouabain had no effect on V_{ba} . Figure 1(B) shows the results of a representative experiment in a different monolayer. At $t = 30$ sec, the basal superfusate was switched from normal Ringers to Ringers plus 0.2 mM ouabain and both V_{ba} and TEP remained unchanged for the next 14 min. Similar results were obtained in two other monolayers where stable intracellular recordings were maintained for more than 10 min. The absence of a change in V_{ba} in response to basal ouabain suggests that the basolateral membrane lacks an electrogenic $\text{Na}^+\text{-K}^+$ pump.

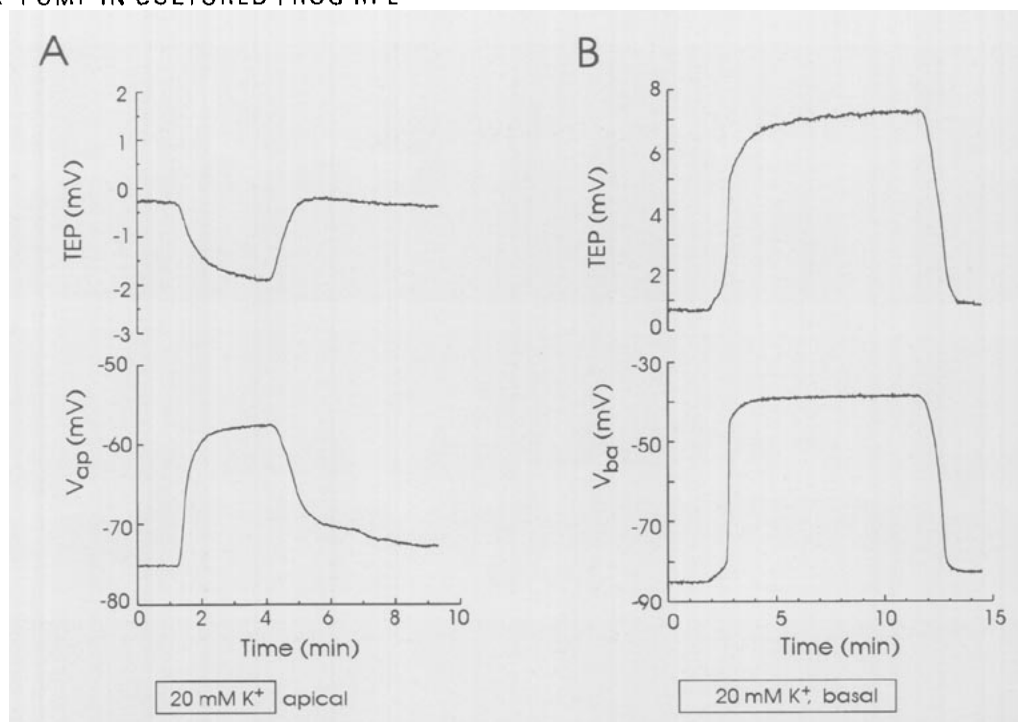


FIG. 2. Apical and basolateral K^+ responses. (A) Apical $[\text{K}^+]$ was increased from 2 to 20 mM (open bar), depolarizing V_{ap} and decreasing TEP. Response was obtained in the same cell as that in Fig. 1(A). (B) Basal $[\text{K}^+]$ was increased from 2 to 20 mM (open bar), depolarizing V_{ba} and increasing TEP. Response was obtained in same cell as that in Fig. 1(B).

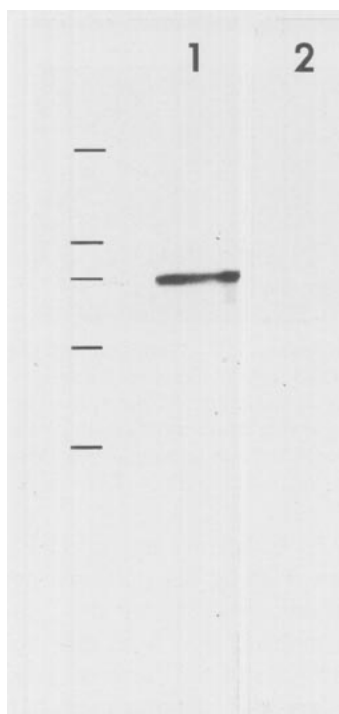


FIG. 3. Immunoblot analysis of pigment epithelium sheets. Whole cell extracts (18 μg of total protein) were gel-fractionated and nitrocellulose replicas probed with rabbit anti- Na^+ , K^+ -ATPase α subunit (31B) (lane 1) or unspecific rabbit IgG (lane 2). Antiserum to $\text{Na}^+\text{-K}^+$ pump protein specifically recognizes a 95-kDa-molecular-weight band. Migration of molecular weight standards (200, 116, 97.4, 66 and 45 kDa) are indicated by dashes at left.

Time course of K^+ diffusion potentials It is possible that the absence of a voltage response to basal ouabain resulted from the failure of the inhibitor to reach the

basolateral membrane because of a diffusion barrier created by the polycarbonate filter and/or its Matrigel coating. To assess the time course of solution composition changes adjacent to the RPE membranes, we measured membrane voltage responses to K^+ concentration ($[\text{K}^+]$) changes in the apical and basal superfusates. Figure 2(A) shows the effect of a ten-fold increase in apical $[\text{K}^+]$ on V_{ap} and TEP. These records were obtained in the same cell as that shown in Fig. 1(A), before exposure to apical ouabain. At the start of the experiment, both sides of the monolayer were superfused with normal Ringers containing 2 mM K^+ , and V_{ap} and TEP measured -75 and -0.3 mV, respectively. At $t = 30$ sec, the apical superfusate was changed to 20 mM K^+ Ringers and, after a 45 sec delay, V_{ap} began to rapidly depolarize, marking the arrival of the wavefront of higher K^+ concentration at the apical membrane. From the flow rate and dead space volume, we estimate that the solution composition change in the apical compartment of the chamber was complete after the first 25–30 sec. The remaining 15–20 sec of the delay probably represents the time it took for K^+ to diffuse through the unstirred layer outside the apical membrane (Miller, Steinberg and Oakley, 1978). Over the next minute, V_{ap} depolarized by 17 mV and TEP decreased by 1.6 mV; the half-time of these voltage changes was approximately 20 sec. In three monolayers, ten-fold increases in apical $[\text{K}^+]$ depolarized V_{ap} by an average of 19.3 ± 3.3 mV and decreased the TEP by 1.4 ± 0.7 mV, with half-times ranging from 6 to 20 sec.

Figure 2(B) shows the responses of V_{ba} and TEP to a ten-fold increase in basal $[\text{K}^+]$. These records were

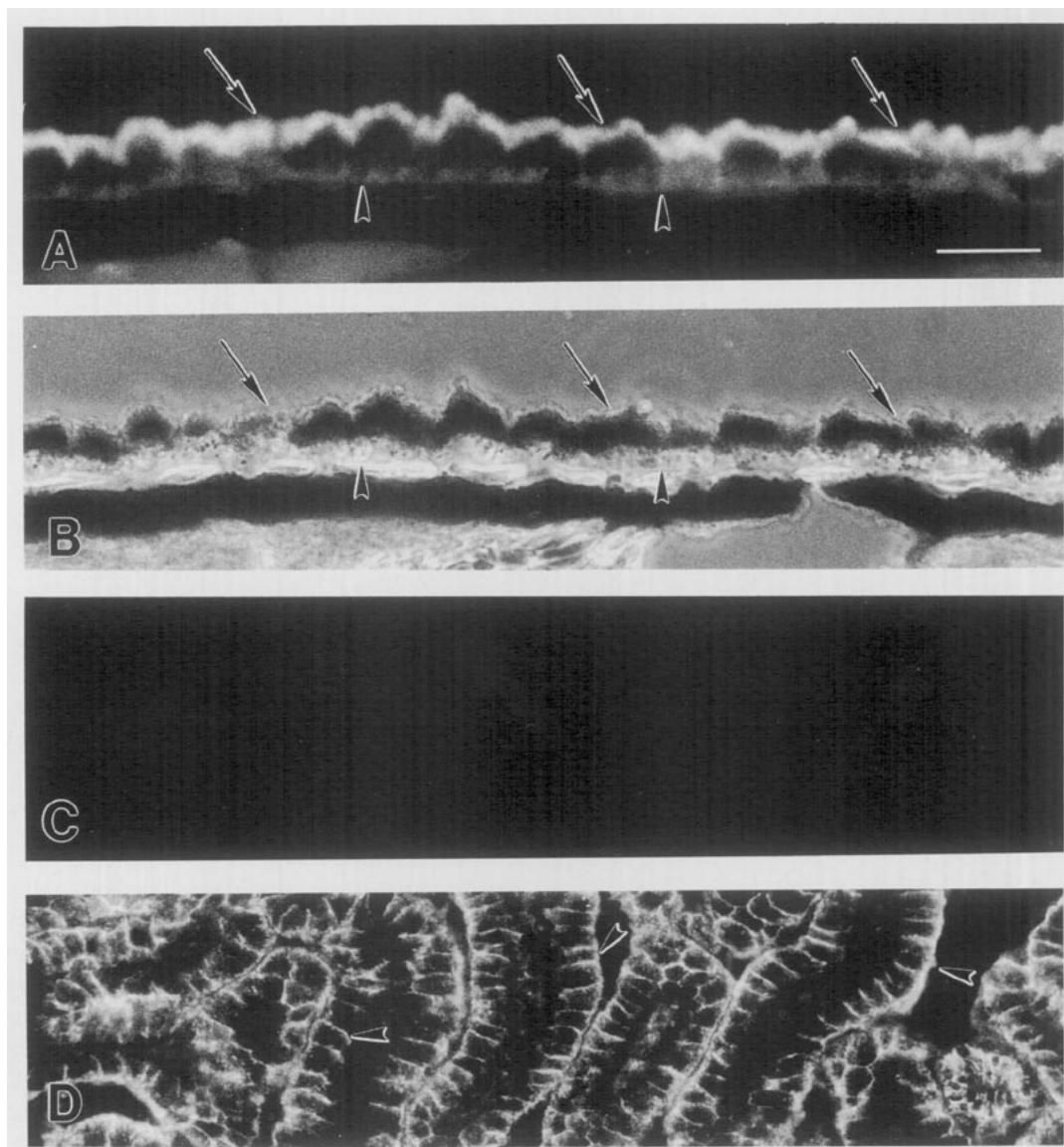


FIG. 4. Distribution of Na^+ , K^+ -ATPase α subunits in tissue sections stained with antibody 31B (A) Eyecup. Intense reaction is seen on the apical surface of RPE cells (B) Phase contrast image of (A). In both panels, microvillous processes are indicated by arrows, while the position of the basal membrane is marked by arrowheads. The apical border of the epithelium appears scalloped due to a small amount of post-mortem contraction. (C) Control showing lack of immunoreaction when the primary antibody is omitted. (D) Kidney. Note that antibody labeling is restricted to the basolateral surfaces of tubule cells (arrowheads). Bar: 50 μm .

obtained in the same cell as that shown in Fig. 1(B). At the start of the experiment, both sides of the monolayer were superfused with normal Ringers (2 mM K^+) and V_{ba} was -85 mV. At $t = 30$ sec, the solution at the basal manifold was changed to 20 mM K^+ Ringers. After a delay of about 70 sec, V_{ba} began to depolarize, presumably marking the arrival of K^+ at the basolateral membrane. The longer delay for the response at the basolateral membrane as compared with that at the apical membrane resulted from a slower perfusion rate and a larger unstirred layer due to the filter and Matrigel substrate that separated the basolateral membrane from the basal compartment of the chamber. Approximately 2 min later, V_{ba} had depolarized to -40 mV and the TEP increased by 6.2 mV; the half-time for these voltage changes was approximately 60 sec. In five monolayers, ten-fold increases in basal

$[\text{K}^+]$ depolarized V_{ba} by an average of 35.1 ± 6.0 mV and increased the TEP by 3.4 ± 1.9 mV, with half-times of 30 to 60 sec.

These results indicate that both the apical and basolateral membranes of the cultured *Xenopus* RPE have relatively large K^+ conductances. They also demonstrate that the polycarbonate filter and Matrigel substrate underlying the monolayer slow, but do not prevent, the diffusion of small solutes from the bulk solution to the basolateral membrane. Assuming that the diffusion coefficient for ouabain is half that for K^+ (Miller et al., 1978), we would expect ouabain to reach the basolateral membrane within 3–5 min from the time the solution was switched at the manifold. Therefore, the lack of a change in V_{ba} in response to basal ouabain indicates that the basolateral membrane has little or no electrogenic Na^+ - K^+ pump activity.

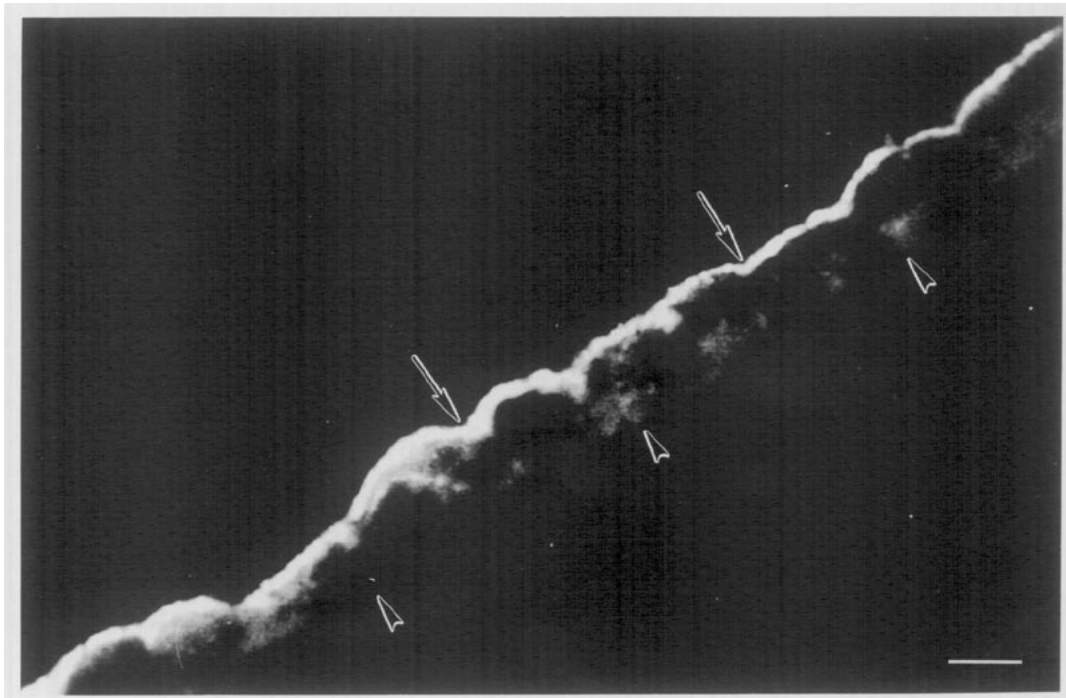


Fig. 5. Subcellular distribution of Na^+ , K^+ -ATPase in cultured RPE. Confocal optical section obtained in a plane parallel to the surface of an immunostained transverse frozen section. Note that strong reaction is present on apical epithelial surfaces (arrows), while lateral and basal membranes (indicated by arrowheads) are relatively unlabeled. Bar: $5\ \mu\text{m}$.

Immunocytochemistry and Immunoblotting

Characterization of anti- Na^+ , K^+ -ATPase The electrophysiological experiments utilizing ouabain inhibition of electrogenic current are capable of detecting only functional ion pumps in the plasma membrane. Therefore, we also examined the distribution of the Na^+ , K^+ -ATPase catalytic component using an antiserum previously shown to be specific for the bovine kidney α subunit (Hieber et al., 1989). Initial characterization of this antibody was carried out by Western blot analysis of detergent extracts obtained from isolated RPE sheets. As shown in Fig. 3 (lane 1), only a single protein band was seen on blots following immunostaining with specific antiserum (31B). This corresponds to an apparent molecular weight of 95 kDa, which is similar to a previous molecular weight determination for the $\alpha 1$ subunit from the *Xenopus* kidney cell line A6 (Verrey et al., 1989). Control blots probed with an unspecific rabbit IgG were entirely devoid of reaction product (Fig. 3, lane 2).

When frozen sections of aldehyde-fixed RPE-choroid-sclera were labeled with primary antiserum followed by secondary antibody conjugated to Texas Red, intense immunoreactivity was observed at the apical border of the pigment epithelium [Fig. 4(A)]. Comparison with the phase contrast image [Fig. 4(B)] indicates that this stained region corresponds to the fringe of microvillous processes. In contrast, very little fluorescence was detected on the basal cell surface, or within the epithelium itself. However, a small amount of antigen may be present on the basolateral membrane, since sections exposed to secondary antibody

alone [Fig. 4(C)], or with an unspecific IgG (data not shown) were completely unreactive. As an additional control, frozen sections of frog kidney were labeled with antibody and examined in the same way. As expected, epithelial cells of the nephron exhibited a polarized distribution of Na^+ , K^+ -ATPase α subunit which was essentially the reverse of that seen with the RPE. Individual cells were labeled on their basal and lateral surfaces only, resulting in a C-shaped staining pattern, while the apical microvillar surface was unlabeled [Fig. 4(D)].

Immunostaining of RPE monolayers Preliminary examination of frozen sections of RPE cultures labeled with specific antiserum indicated that Na^+ , K^+ -ATPase α subunits were highly polarized, in a manner very similar to the epithelium in situ (data not shown). However, better spatial resolution was obtained when specimens were examined by confocal microscopy to eliminate blur. Figure 5 represents an optical slice obtained parallel to the cut edge of the $10\text{-}\mu\text{m}$ frozen section reacted with antiserum 31B. As with the native epithelium, antibody labeling was confined to the apical cell surface. There was essentially no staining of lateral intercellular membranes, or the basal surface where cells contact the Matrigel substrate. In separate immunocytochemical experiments, we have demonstrated specific labeling of basal membranes in these monolayers (Chen and Defoe, manuscript in preparation). Thus, we believe that the lack of basolateral staining with antiserum 31B is not due to restricted access of these reagents to antigen. To view the distribution of Na^+ , K^+ -ATPase within individual cells, intact cultures were immunostained

and, after flat-mounting, a series of horizontal optical sections (x - y scans) were collected in the z -axis through selected areas of the monolayer (Fig. 6). In x - y scans through the most apical portions of cells, a punctate labeling pattern was seen, consistent with staining of microvillous processes [Fig. 6(A)]. On the other hand, virtually no staining was observed in the middle or lower one-third of most of the cells in Fig. 6(B). The faint reaction seen near intercellular borders in some regions is due to a small amount of residual apical labeling. Because of differences in the heights of individual cells in the monolayer, this plane of optical section intersects both apical and lateral membranes.

4. Discussion

Membrane Parameters

Although there is no published information regarding the electrophysiological properties of native *Xenopus* RPE, there is ample data on the bullfrog RPE-choroid to which the present findings can be compared (Miller and Steinberg, 1977; Miller et al., 1978; Hughes et al., 1988). The apical membrane potential of cultured *Xenopus* RPE monolayers averaged -76 mV, compared to -88 mV in bullfrog RPE explants (Miller and Steinberg, 1977). The lower value of V_{ap} in the cultured *Xenopus* RPE might be due in part to a low shunt resistance (R_s), which would cause V_{ap} to be depolarized to a greater extent by the basolateral membrane EMF. The ratio of apical to basolateral membrane resistances (R_{ap}/R_{ba}) averaged 0.34, which is comparable to that reported for bullfrog RPE explants (0.42; Miller and Steinberg, 1977).

The transepithelial voltage of cultured monolayers was near zero in the steady state. In contrast, the TEP of bullfrog RPE explants is in the range from 10 to 20 mV. The most likely explanation for the low TEP in the cultured monolayers is a low R_s , due to edge damage under the sealing ridges of the chamber, or to cells missing from the monolayer. The possibility of a low R_s is supported by the low R_t ($\sim 100 \Omega \text{ cm}^2$) of the mounted monolayers and by the observation that ouabain inhibition of the apical Na^+ - K^+ pump current produced voltage changes of similar magnitude at both the apical and basolateral membranes (see eqns 1 and 2). The electrical coupling of the two membranes, however, did not prevent us from localizing the origin of the Na^+ - K^+ pump current to the apical or basolateral membrane because the recording chamber allowed the two membranes to be exposed to ouabain independently.

Membrane Location of the Electrogenic Na^+ - K^+ Pump

In a previous study (Miller et al., 1978), the electrogenicity of the apical Na^+ - K^+ pump in the bullfrog RPE was demonstrated by showing that the onset of the initial fast phase of V_{ap} depolarization

coincided with the arrival of ouabain to the apical membrane. This fast phase was followed by a slower phase of depolarization that was attributed to the rundown of ionic gradients across both membranes. Recent studies in bovine and human RPE-choroid preparations have shown a similar sequence of ouabain-induced voltage changes (Joseph and Miller, 1991; Quinn and Miller, 1992). In the present study, we found that apical ouabain produced a biphasic membrane voltage response in cultured *Xenopus* RPE, confirming the presence of an electrogenic Na^+ - K^+ pump in the apical membrane.

Other investigators have tested the effect of basal ouabain on V_{ba} in human and bovine RPE explants and have concluded from the absence of any voltage change that the basolateral membrane lacks an electrogenic Na^+ - K^+ pump (Joseph and Miller, 1991; Quinn and Miller, 1992). In contrast to RPE explants, there is electrophysiological evidence that cultured human RPE grown on permeable substrates expresses electrogenic Na^+ - K^+ pumps on both apical and basolateral membranes (Hernandez et al., 1993; Hu et al., 1993). The present study shows that cultured *Xenopus* RPE monolayers retain a higher degree of polarity, with the electrogenic Na^+ - K^+ pump restricted to the apical membrane.

Polarity of the Na^+ , K^+ -ATPase Subunits

As with functional studies, there is substantial anatomical evidence for apical polarity of the Na^+ , K^+ -ATPase in native RPE. This was first established in experiments using autoradiography or tissue microdissection to localize ^3H -ouabain binding (Bok, 1982; Ostwald and Steinberg, 1980). More recent immunocytochemical studies have confirmed the fact that both the catalytic α subunit and glycosylated β subunit of the enzyme complex are largely restricted to apical membrane domains *in situ* (Okami et al., 1990; Gunderson et al., 1991). While evidence indicates that basolateral α subunits are present (Okami et al., 1990), the substantially greater membrane area of the apical plasmalemma clearly indicates that the bulk of the protein must be located here.

In contrast to the native epithelium, cultured RPE Na^+ , K^+ -ATPase subunits have until now been shown to be unpolarized. In an initial study, Rizzolo (1990) used an antibody to the glycosylated β subunit to examine protein distribution in embryonic chicken pigment epithelium. Despite the fact that monolayers mimicked many of the ultrastructural features of the native epithelium, staining was found in both apical and basolateral membranes. Furthermore, while plating dispersed cells on their natural basement membrane was effective in promoting the basal distribution of β_1 integrin, such conditions had little effect on polarity of the Na^+ - K^+ pump (Rizzolo, 1991). More recently, Gunderson et al. (1991) have obtained qualitative evidence for apical localization of α and β

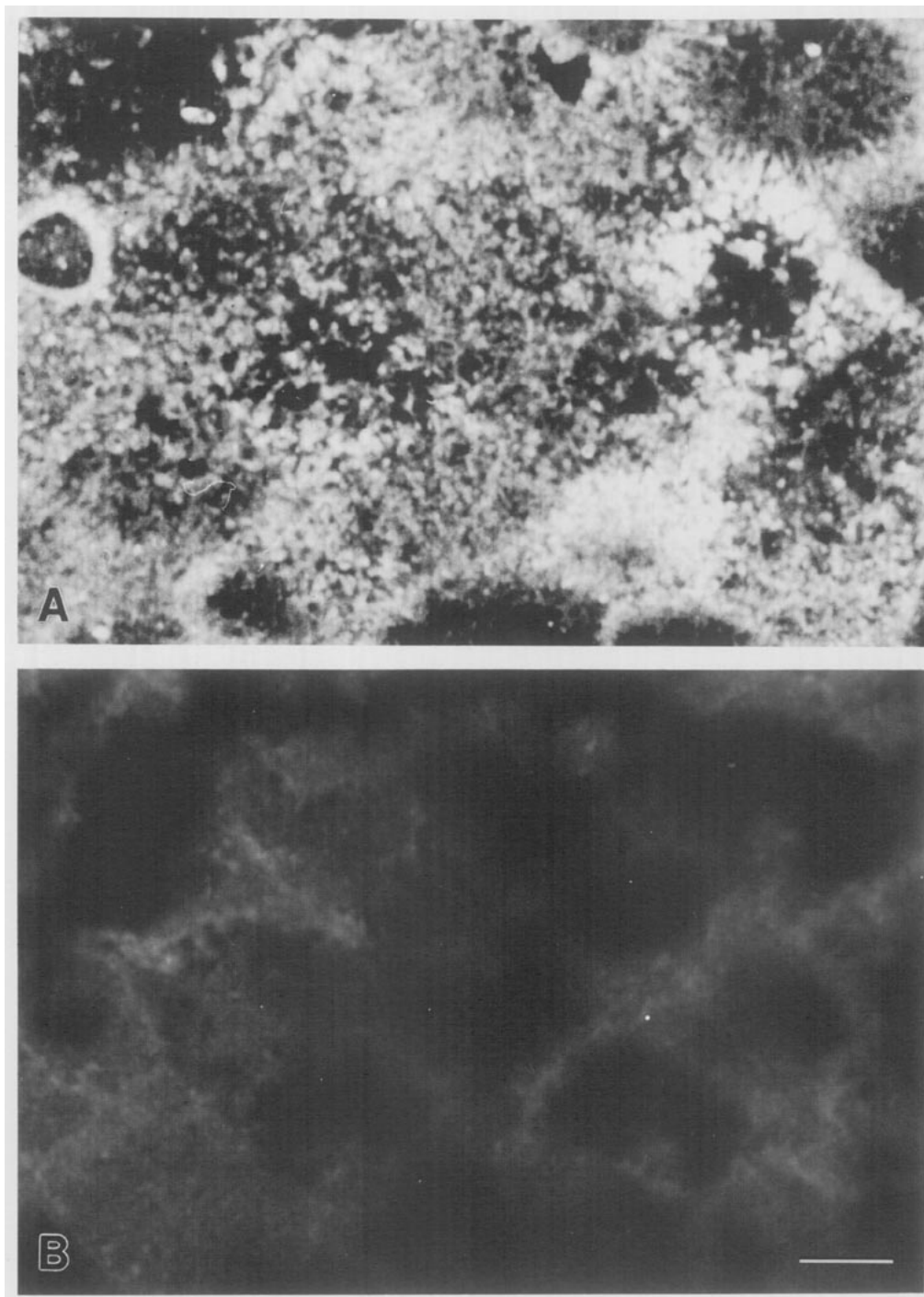


FIG. 6. Localization of Na^+ , K^+ -ATPase in cultured RPE using confocal microscopy. (A) and (B) represent horizontal x-y scans of the same cell group in a stained monolayer flat-mount at two different levels. A punctate labeling pattern, representing stained microvilli, is visualized at the apical surface of the cells (A). (B) An optical section through the middle portion of the monolayer (scan 28, $\sim 14 \mu\text{m}$ from microvillous border) shows limited reaction. The weak reaction seen at cell boundaries is labeling of the apical surface, which is indented (see Fig. 5). A longer photographic exposure was used in order to show the outlines of cells, and thus to aid in comparison of the two images. Bar: $5 \mu\text{m}$.

subunits of the enzyme in primary cultures of neonatal rat RPE. However, these cultures do not form continuous monolayers on filter supports (Gundersen et al., 1991), precluding quantitative determination of pump distribution using physiological techniques.

Our immunocytochemical results localize Na^+ , K^+ -ATPase catalytic subunits to the apical surface of RPE cultures, a pattern that is very similar to that seen in

the epithelium *in situ*. This indicates that monolayers are capable of maintaining a polarized enzyme distribution in the absence of interactions with the neural retina. Furthermore, in this study we have been able, for the first time, to correlate functional polarity with anatomical polarity in cultured cells. The immunolocalization data are in close agreement with the electrophysiological determination of functional

Na⁺-K⁺ pumps. Thus, within the limits of sensitivity of the techniques used, this implies that substantial amounts of inactive enzyme are not present in the basolateral membrane.

These initial studies examining primary RPE cultures show that a crucial component of the epithelial transport pathway is polarized in an appropriate fashion. It will be important to investigate the distribution of other components, including specific ion channels and pumps, in order to demonstrate intact ion and fluid transport pathways in these cells. Such a model system should be useful not only for studies of epithelial physiology, but also for examination of RPE-retina interactions.

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