Effect of coupling on volume-regulatory response of ciliary epithelial cells suggests mechanism for secretion

V. E. WALKER, J. W. STELLING, H. E. MILEY, AND T. J. C. JACOB
School of Molecular and Medical Biosciences, University of Wales, Cardiff CF1 3US, United Kingdom

Walker, V. E., J. W. Stelling, H. E. Miley, and T. J. C. Jacob. Effect of coupling on volume-regulatory response of ciliary epithelial cells suggests mechanism for secretion. Am. J. Physiol. 276 (Cell Physiol. 45): C1432–C1438, 1999.—The ciliary epithelium of the eye secretes the aqueous humor. It is a double epithelium arranged so that the apical surfaces of the nonpigmented ciliary epithelial (NPCE) and pigmented ciliary epithelial (PCE) cells face each other and the basolateral membranes face the inside of the eye and the blood, respectively. We have investigated the volume responses of both single cells and coupled pairs from this tissue to osmotic challenge. Both NPCE and PCE cells undergo regulatory volume increase (RVI) and decrease (RVD) when exposed to hyper- and hypovolemic solution, respectively. In hypovolemic solution single cells swell and return to their original volumes within ~3 min. In nonpigmented cells RVD could be inhibited by blockers of volume-activated Cl− channels (tamoxifen (100%) > quinidine (87%) > DIDS (84%) > 5-nitro-2-(3-phenylpropylamino)benzoic acid (80%) > SITS (58%) and K+ channels [Ba2+ (31%)]. However, in PCE cells these inhibitors and additionally tetraethylammonium and Gd3+ were without effect. Only bumetanide, an inhibitor of Na+–K+–2Cl− cotransport, was found to have any effect on RVD in PCE cells. NPCE-PCE cell coupled pairs also underwent RVD, but with altered kinetics. The onset of RVD of the PCE cell in a pair occurred ~80 s before that of the NPCE cell, and the peak swell was reduced. This is consistent with fluid movement from the PCE to the NPCE cell. The effect of the volume-activated Cl− channel inhibitor tamoxifen was to eliminate this difference in the times of onset of RVD in coupled cell pairs and to inhibit RVD in both the NPCE and PCE cells partially. On the basis of these observations we suggest that fluid is transferred from the PCE to the NPCE cell in coupled pairs during cell swelling and the subsequent RVD. Furthermore, we speculate that reciprocal RVI–RVD could underlie aqueous humor secretion.

ciliary epithelium; secretion; fluid transport; volume regulation; ion channels

IT HAS BEEN SUGGESTED (1) that the same mechanisms that underlie volume regulation may also subserve fluid secretion. Of the mechanisms that are involved in volume regulation, those involved in regulatory volume decrease (RVD), e.g., volume-activated K+ and Cl− channels, result in solute efflux, whereas those involved in regulatory volume increase (RVI), e.g., the Na+–K+–2Cl− cotransporter, result in solute influx (see Ref. 5 for a review). If these mechanisms were sited on opposite membranes in epithelia, then this arrangement would provide a mechanism for the vectorial transport of solute, and hence water. With the uptake mechanisms situated on the basolateral (blood) side and the efflux or secretory mechanisms situated on the apical (mucosal) membrane, fluid secretion would be achieved; conversely, with uptake mechanisms situated on the apical membrane and efflux mechanisms on the basolateral membrane, fluid absorption would be accomplished.

In the ciliary epithelium of the eye, which is, uniquely, a bilayered epithelium with the two epithelial cell layers apposed at their apical membranes, the uptake and efflux mechanisms could be differentially segregated into different cells; if this were the case, a vectorial movement of fluid could be achieved. There is evidence that the two cell types have different properties with respect to their complement of ion channels (7, 10, 17, 18). The apical membranes of the two cells are effectively short-circuited by communicating gap junctions (3, 11, 13), allowing solute and water movement between the two cell layers in the ciliary epithelium. Therefore, following the suggestion of Civan et al. (1), we decided to investigate volume regulation in these cells and look for evidence of vectorial fluid movement.

METHODS

Dissection and cell culture. Tips of ciliary processes were dissected from bovine eyes (obtained from a local abattoir) within 1–3 h post mortem. Cells were isolated with 0.25% trypsin-0.02% EDTA in PBS at 35°C for 20 min in a shaking water bath, a method previously described by Jacob (6). The incubation mixture was then triturated with a Pasteur pipette to break up clumps and allowed to settle, and the supernatant was decanted, spun at 1,000 rpm, resuspended in HEPES-buffered culture medium (E199; Sigma, Poole, UK) with 10% FCS (Sigma) twice, and finally plated on glass coverslips. Cells were incubated overnight in E199 plus 10% FCS in a humidified incubator (Gallenkamp, Loughborough, UK) at 37°C.

Electrical recording. Cells attached to coverslips were transferred to the recording chamber on the stage of an inverted fluorescence microscope (Leitz DM1L; Leica, Milton Keynes, UK). Cell pairs of pigmented ciliary epithelial (PCE) and nonpigmented ciliary epithelial (NPCE) cells were selected and patch clamped (see Ref. 14 for a detailed description) with electrodes (4–8 MΩ; pulled on a PB-7 electrode puller; Narashige, Tokyo, Japan) containing intracellular buffer (see Solutions) and were bathed in bath solution A (see Solutions). The cells were whole cell patch-clamped [using either a Dagan 8900 or List (Darmstadt, Germany) EPC-7 amplifier] as described previously (14) and recorded in current clamp.

Digital image recording and image analysis. Images of single cells and coupled pairs of cells were recorded every 20 s with a charge-coupled device (CCD) camera (EDC-1000HR; Electrim) mounted on an inverted microscope (Leitz DM-1L;
Solutions. Pipette solution, used for fluorescent dye coupling experiments, contained 1 mM Lucifer yellow and (in mM) 6 NaCl, 56 KCl, 84 potassium gluconate, 1.1 EGTA, 10 HEPES, 2 MgCl₂, 10⁻⁵ CaCl₂, and 20 sucrose and was adjusted to pH 7.25 with 1 M NaOH.

For experiments at 22°C the solution was composed of (in mM) 125 NaCl, 5 KCl, 10 HEPES, 10 NaHCO₃, 0.5 MgCl₂, 2 CaCl₂, 5 glucose, and 20 sucrose and was adjusted to pH 7.4 with 1 M NaOH (solution A). For experiments at 35°C the solution was composed of (in mM) 90 NaCl, 4.4 KCl, 26 NaHCO₃, 10 HEPES, 2 CaCl₂, 0.5 MgCl₂, 5 glucose, and 20 sucrose and was adjusted to pH 7.4 with 1 M NaOH (solution B). Solutions were made hypotonic by the addition of 50% distilled water and were made hypertonic by the addition of 100 mM sucrose.

All chemicals were obtained from Sigma. The osmolarities of all the solutions were measured by depression of the freezing point using an osmometer (Osmomat 30; Gonotec) and were within ±10 mosmol/l of the theoretical values: isotonic, hypotonic, and hypertonic solution osmolarities were 300, 150, and 400 mosmol/l, respectively.

Statistics. Statistical analysis of the data was achieved with Student’s two-tailed t-test.

RESULTS

RVD in single cells. Using a CCD camera mounted on an inverted microscope, we recorded digital images of both single and coupled pairs of cells. Figure 1 shows a coupled pair of cells in isotonic solution (Fig. 1A), 140 s after exposure to 50% hypotonic solution (Fig. 1B), and 600 s after exposure to hypotonic solution (Fig. 1C). Individually, both cells of the ciliary epithelium, the PCE and NPCE cells, are capable of RVD and RVI when exposed to hypo- and hypertonic solutions, respectively (9, 9a, 12) (Fig. 2). When exposed to 50% hypotonic solution, the NPCE and PCE cells swelled by 54 ± 17 (n = 24) and 60 ± 7% (n = 30), respectively. The times to peak volume were 52.5 ± 6.9 s (n = 24) for the nonpigmented cells and 44.7 ± 5.7 s (n = 30) for the pigmented cells. In the absence of inhibitor, volume-regulatory mechanisms overtook the swelling process and caused a decrease in volume. As illustrated in Fig. 2, both cell types had completely recovered their former volumes while still exposed to hypotonic solution. The time constants (the times for the volumes to reach 1/e of the total response) for this RVD, obtained by fitting a single-exponential function to the volume decrease, were 121.7 ± 26.9 s (n = 3) for NPCE cells and 99.5 ± 15.3 s (n = 4) for PCE cells (Table 1).

RVI. Both NPCE and PCE cells increased their volumes in response to hypertonic solutions and then underwent RVI (Fig. 2). The peak shrink values were 32 ± 4% (n = 4) for NPCE cells and 34 ± 5% (n = 6) for PCE cells. The time constants of the subsequent RVI were 9.4 ± 2.2 min (n = 4) for NPCE cells and 33.9 ± 24.7 min (n = 6) for PCE cells (Table 2). These experiments were carried out at 35°C. RVI was not observed at room temperature.

Effect of temperature on RVD. There was no major effect of temperature on either the peak swell or time constant of RVD for NPCE or PCE cells. At 37°C the time constants of RVD were 88.8 ± 26.2 s (n = 4) for NPCE cells and 125.4 ± 35.4 s (n = 7) for PCE cells and the peak swell values (relative volume) were 1.62 ± 0.23 (n = 5) for NPCE cells and 1.68 ± 0.11 (n = 11) for PCE cells. These values are not significantly different from those at 37°C (see Table 1).

Effect of inhibitors on RVD. In an attempt to identify some of the mechanisms underlying RVD, we used a range of different inhibitors. In particular we chose inhibitors that are known to be more or less selective

![Fig. 1. Cell swelling in a pigmented ciliary epithelial (PCE)-nonpigmented ciliary epithelial (NPCE) coupled pair of cells. Digital images were taken before cell swelling (A); at peak of cell swelling, 200 s after addition of hypotonic solution (B); and after regulatory volume decrease (RVD), 15 min after addition of hypotonic solution (C). Scale bar = 10 µm.](http://ajpcell.physiology.org/)
for Cl\(^{-}\) and K\(^{+}\) channels. To block Cl\(^{-}\) channels, we used DIDS, SITS, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), quinidine, bumetanide, and tamoxifen; to block K\(^{+}\) channels, we used Ba\(^{2+}\), quinidine, and tetraethylammonium (TEA; in PCE cells). All of these inhibitors prevented RVD to a greater or lesser extent in NPCE cells (Fig. 3). The degrees of inhibition, calculated from the ratio of volume recovery to total volume increase, were as follows: 100% (tamoxifen), 87% (quinidine), 84% (DIDS), 80% (NPPB), 77% (butametanide), 58% (SITS), and 31% (Ba\(^{2+}\)). But, of all the inhibitors, and additionally Gd\(^{3+}\) and TEA, only bumetanide caused a significant block (54%) of RVD in PCE cells, prolonging the time constant for the incomplete RVD from a control value of 123 s to 212 s (Fig. 3).

A summary of the results of the inhibitor studies is given in Table 3.

The inhibitors, with the exception of bumetanide, were ineffective in preventing RVD in the PCE cells. The two K\(^{+}\) channel blockers, Ba\(^{2+}\) and quinidine, delayed the onset of RVD by 195 ± 13 (n = 4) and 130 ± 17 s (n = 4), respectively, without actually preventing RVD, which went to completion in both cases.

Coupled cell pairs: injection of Lucifer yellow. To examine the behavior of coupled (NPCE-PCE) cell pairs, we demonstrated that the cells were coupled by injecting one cell of the pair with Lucifer yellow. Figure 4 illustrates a light micrograph (A) of a coupled pair, and the same pair under fluorescence illumination after dye injection (B). Lucifer yellow was injected into the PCE cell of a cell pair, and the dye spread from the site of injection into the NPCE cell, demonstrating that the cells form a coupled pair.

Coupled cell pairs: swelling and RVD. Coupled NPCE-PCE cell pairs were exposed to 50% hypotonic solution, and the volumes of both cells were determined as described for single cells. The PCE cells swelled less (11 ± 2%; n = 5) than their NPCE cell partners (33 ± 7%; n = 5) and also less than their uncoupled counterparts (30 ± 4%; n = 4) (Fig. 5). This reduced swelling is significantly different (P = 0.0094; 2-tailed t-test) from the mean swelling for PCE cells (60 ± 7%; n = 30). The coupled NPCE cells and their uncoupled counterparts swell to the same extent: 33 ± 7% (n = 5) compared with 30 ± 4% (n = 3). Even when efflux is blocked by a range of inhibitors, the peak swell is more or less the same as the control value (Table 3), perhaps indicating that mechanical factors (e.g., cytoskeleton, membrane elasticity) set an upper limit on the cell volume.

Coupled NPCE cells reached their peak volumes 80 s after the PCE cells (Fig. 5); the time to peak volume after exposure to hypotonic solution was 164 ± 23 s

Table 1. Regulatory volume decrease

<table>
<thead>
<tr>
<th>Coupled Pairs</th>
<th>Single Cells</th>
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<tbody>
<tr>
<td>NPCE</td>
<td>PCE</td>
</tr>
<tr>
<td>Peak swell, relative volume</td>
<td>1.33 ± 0.07 (5)</td>
</tr>
<tr>
<td>Time constant, s</td>
<td>184.6 ± 35.7 (5)</td>
</tr>
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</table>

Values are means ± SE for nos. of experiments in parentheses. Time constants were obtained by fitting a single-exponential function to data. Experiments were performed at 37°C (see METHODS for details). NPCE, nonpigmented ciliary epithelial cells; PCE, pigmented ciliary epithelial cells.
Table 2. Regulatory volume increase

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<tr>
<th></th>
<th>Coupled Pairs</th>
<th>Single Cells</th>
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<tbody>
<tr>
<td></td>
<td>NPCE</td>
<td>PCE</td>
</tr>
<tr>
<td>Peak shrink, relative volume</td>
<td>0.79 ± 0.04 (3)</td>
<td>0.89 ± 0.03 (3)</td>
</tr>
<tr>
<td>Time constant, min</td>
<td>43.4 ± 39.7 (3)</td>
<td>226 ± 198 (3)</td>
</tr>
</tbody>
</table>

Values are means ± SE for nos. of experiments in parentheses. Time constants were obtained by fitting a single-exponential function to data. Experiments were performed at 37°C (see METHODS for details).

(n = 5) for coupled PCE cells, significantly different (P = 0.0474) from the value for coupled NPCE cells, 204 ± 11.7 s (n = 5). For uncoupled cells the times to peak volume were 193.3 ± 17.6 s (n = 3) for PCE cells and 180.0 ± 30.6 s (n = 34) for NPCE cells; these values are not significantly different.

The time constants of RVD, 184.6 ± 35.7 s (n = 5) for NPCE cells and 130.9 ± 74.7 s (n = 5) for PCE cells, were not significantly different (P = 0.5348).

Effect of tamoxifen on RVD in coupled pairs. When coupled pairs of NPCE and PCE cells were exposed to hypotonic solution in the presence of tamoxifen (10⁻⁵ M; Fig. 6) three things were noted. First, RVD in the NPCE cells was no longer 100% inhibited as in single cells; instead the inhibition was 48.1 ± 14.4% (n = 7). Second, RVD was inhibited in the PCE cells by 33.7 ± 12.3% (n = 7), whereas tamoxifen had no effect on RVD in single PCE cells. Third, the peak of the swelling for PCE cells was no longer time shifted. The time to peak volume was 171.4 ± 36.3 s (n = 7) for PCE cells and 202.9 ± 42.6 s (n = 7) for NPCE cells. These values are not significantly different from those for single cells.

Fig. 3. Inhibition of RVD in single NPCE and PCE cells. Effects of inhibitors of Cl⁻ channels on RVD in response to 50% hypotonic solution in single NPCE cells (○) and PCE cells (●) were investigated. A: 1 mM DIDS; B: 1 mM SITS; C: 10⁻⁴ M 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB); D: 1 mM quinidine; E: 10⁻⁴ M bumetanide; F: 10⁻⁵ M tamoxifen. Data are means ± SE (vertical lines) from no. of experiments given in Table 3. All inhibitor experiments were carried out at room temperature.
DISCUSSION

In this study we have determined the characteristics of volume regulation in PCE and NPCE cells. From the degree of volume change, the time to peak or minimum volume, and the time constant of RVD or RVI, we can define the kinetics of the processes of RVI and RVD. We have done this for PCE and NPCE cells under two different conditions: as single cells and as coupled, heterogeneous (NPCE-PCE cell) pairs.

We have discovered two things: first, NPCE and PCE cells have different RVD mechanisms as defined by their different inhibitor sensitivities; second, coupling significantly alters the properties of RVD in a way that suggests vectorial solute movement from PCE to NPCE cells.

Single-cell studies. The kinetic parameters for RVD in single PCE and NPCE cells are similar (Table 1). Differences between the time constants of RVD in our studies (<100 s) and those of other laboratories (<10 min; Refs. 1 and 3) are due to the indirect measurement technique used in the latter studies, a fact that has been discussed elsewhere (9). Farahbakhsh and Fain (4) measured RVD directly by interference contrast microscopy and found time constants for rabbit NPCE cells similar to those reported in this study.

The pharmacologies of the mechanisms of RVD are radically different between the two cell types (8). All the Cl⁻ channel inhibitors we used (DIDS, SITS, NPPB, quinidine, and tamoxifen) inhibited RVD in the NPCE cells to a greater or lesser extent, but none of them had any effect on RVD in the PCE cells. Mitchell et al. (12) reported that, of the Cl⁻ channel inhibitors that blocked the volume-activated Cl⁻ current in NPCE cells, DIDS, SITS, NPPB, dideoxyforskolin, verapamil, tamoxifen, and quinidine (16), only DIDS, SITS, and NPPB had any effect on PCE cells. These three inhibitors operate a voltage-dependent block because of their negative charge. Mitchell et al. (12) predicted that the small degree of block with DIDS and SITS at negative potentials would render them almost ineffective at the resting membrane potential, and this was found to be the case. Further support for the difference between the NPCE and PCE cells comes from the finding of different populations of volume-activated Cl⁻ channels in the two cell types (18).

The K⁺ channel inhibitor Ba²⁺ also failed to inhibit RVD in the PCE cells, as did Gd³⁺, a nonselective cation channel inhibitor. The process of RVD in these cells was not, however, so robust as to be uninhibitable. One

Table 3. Inhibition of regulatory volume decrease in single cells

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<tr>
<th>Inhibitor</th>
<th>Nonpigmented</th>
<th>Pigmented</th>
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<tr>
<td></td>
<td>𝜏, s Peak swell %Block</td>
<td>𝜏, s Peak swell %Block</td>
</tr>
<tr>
<td>DIDS</td>
<td>2,000</td>
<td>1.54 ± 0.14 (4) 83.9 ± 9.3</td>
</tr>
<tr>
<td>SITS</td>
<td>526</td>
<td>1.68 ± 0.13 (3) 58.2 ± 9.1</td>
</tr>
<tr>
<td>Bumetanide</td>
<td>909</td>
<td>1.75 ± 0.11 (4) 76.9 ± 13.9</td>
</tr>
<tr>
<td>NPPB</td>
<td>1,667</td>
<td>1.74 ± 0.09 (4) 80.4 ± 16.9</td>
</tr>
<tr>
<td>Quinidine</td>
<td>1,429</td>
<td>2.43 ± 0.76 (3) 86.7 ± 13.3</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>ω</td>
<td>1.39 ± 0.01 (3) 100</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>667</td>
<td>1.67 ± 0.04 (3) 30.9 ± 15.5</td>
</tr>
<tr>
<td>TEA</td>
<td></td>
<td></td>
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<td>Gd³⁺</td>
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Values are means ± SE (for nos. of experiments in parentheses for peak swell). Time constants (𝜏) were obtained by a single-exponential fit to mean data. Percent block was obtained from following formula: %block = [1 – (volume recovery/total volume increase)] × 100.

Concentrations of inhibitors were as follows: DIDS, 1 mM; SITS, 1 mM; bumetanide, 10⁻⁴ M; 5-nitro-2-(3-phenylpropylaminobenzoic acid (NPPB), 10⁻⁴ M; quinidine, 1 mM; tamoxifen, 10⁻⁵ M; Ba²⁺, 2 mM; tetraethylammonium (TEA), 10 mM; Gd³⁺, 3 mM; ω = infinite.

Fig. 4. Dye-coupled PCE-NPCE cell pair. A: phase-contrast image of ciliary epithelial cells. A cell pair consisting of a PCE cell (lower) and NPCE cell (upper) in top left quadrant of picture was injected with Lucifer yellow via a patch electrode attached to pigmented cell. B: fluorescence image of same cells, demonstrating that Lucifer yellow, injected into PCE cell, is able to diffuse into NPCE cell via gap junctions. Scale bar = 10 µm.
inhibitor that affected RVD in the PCE cells was bumetanide, a blocker of Na\(^+\)-K\(^+\)-Cl\(^-\) cotransport. It is thought that this was achieved by a depletion of intracellular Cl\(^-\), reducing the gradient for Cl\(^-\) efflux during RVD.

RVD in coupled pairs of NPCE-PCE cells. In the ciliary epithelium the NPCE and PCE cells form a syncytium. We used coupled pairs of NPCE and PCE cells, first described by Edelman et al. (3), as a model for the ciliary epithelium. To demonstrate that they were functionally coupled, we injected Lucifer yellow into the pigmented cell of such a pair and observed that the dye spread into the nonpigmented cell.

The coupled NPCE-PCE cell pair responded quite differently to hypotonic challenge than did single NPCE and PCE cells. Two facts emerge from this experiment.

1. The peak swell of the single pigmented cells becomes very much smaller when the cells are coupled; when the cells were uncoupled, the peak swell values for NPCE and PCE cells were not significantly different (54 ± 17%, n = 24, and 60 ± 7%, n = 30, respectively).

2. The time of peak swell for the pigmented cells, which is more or less coincident with that for the nonpigmented cells when they are separate, precedes that for the nonpigmented cells by 80 s when the cells are coupled (Fig. 5, arrows). We interpret these observations as indicating a flow of fluid from the pigmented to the nonpigmented cells. The PCE cells begin to swell, but, before they reach their peak volume, solute moves into the NPCE cells and exits via channels in the NPCE cells. The question of
what drives the movement of fluid from PCE to NPCE cells is an interesting one. There are a number of possibilities. One is that the PCE cells could have a lower water permeability than the NPCE cells, so that the concentration gradient drives solute from PCE to NPCE cells. This possibility seems implausible given the observation that the times to peak swelling for uncoupled NPCE and PCE cells were the same. Alternatively, the structural union of the two cells may in some way alter the membrane transport properties of the PCE cells. The physics of surface tension offers another explanation. At equal surface tension a smaller bubble has a larger internal pressure than a bigger one, so its contents will be pumped into the larger bubble when they communicate.

The two cell types clearly have very different RVD mechanisms, which may have different activation properties or set points, such that more solute per unit time can pass through the NPCE membranes, resulting in a net movement of fluid from PCE to NPCE cells. The addition of tamoxifen, which blocks RVD in NPCE but not PCE cells, causes an apparently reduced RVD in both cell types. The time course of the RVD in NPCE cells follows that in the PCE cells. This could be interpreted as a movement of solute from NPCE cells (now efflux inhibited) to PCE cells, where it passes through tamoxifen-resistant pathways.

What relevance do these osmotically induced fluxes have to fluid transport? We induced cell swelling by lowering the external osmolarity, but the same effect could be obtained by increasing the intracellular osmolarity. This could be achieved by uptake mechanisms such as cotransporters (2, 15). Such mechanisms are known to be involved with RVI. Figure 7 illustrates a model in which volume oscillation is achieved by having the volumes at which RVI and RVD turn on and off slightly greater and smaller than the "set" volume. The cyclical and reciprocal activation of RVI and RVD mechanisms, situated on opposite membranes, would enable an oscillatory fluid flow across the tissue without the need for changes in external osmolarity.

In conclusion, we show that vectorial flow from one cell to another can be achieved under conditions of osmotic swelling and may be due to differences in the volume-activated ion channels between the two cell types. For a given osmotic challenge NPCE cells pass more solute than PCE cells. This creates a movement of solute from PCE to NPCE cells, via communicating gap junctions. Because the PCE cells are on the serosal (blood) side of the ciliary epithelium and the NPCE cells face the inside of the eye, this vectorial flow is in the same direction as that for the secretion of aqueous humor.

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Address for reprint requests and other correspondence: T. J. C. Jacob, School of Biosciences, Cardiff Univ., Cardiff CF1 3US, UK (E-mail: jacob@cardiff.ac.uk).

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