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The Role of the Tight Junction in Paracellular Fluid Transport across Corneal Endothelium. Electro-osmosis as a Driving Force

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Abstract. The mechanism of epithelial fluid transport is controversial and remains unsolved. Experimental difficulties pose obstacles for work on a complex phenomenon in delicate tissues. However, the corneal endothelium is a relatively simple system to which powerful experimental tools can be applied. In recent years our laboratory has developed experimental evidence and theoretical insights that illuminate the mechanism of fluid transport across this leaky epithelium. Our evidence points to fluid being transported via the paracellular route by a mechanism requiring junctional integrity, which we attribute to electro-osmotic coupling at the junctions. Fluid movements can be produced by electrical currents. The direction of the movement can be reversed by current reversal or by changing junctional electrical charges by polylysine. Aquaporin 1 (AQP1) is the only AQP present in these cells, and its deletion in AQP1 null mice significantly affects cell osmotic permeability but not fluid transport, which militates against the presence of sizable water movements across the cell. By contrast, AQP1 null mice cells have reduced regulatory volume decrease (only 60% of control), which suggests a possible involvement of AQP1 in either the function or the expression of volume-sensitive membrane channels/transporters. A mathematical model of corneal endothelium predicts experimental results only when based on paracellular electro-osmosis, and not when transcellular local osmosis is assumed instead.
Our experimental findings in corneal endothelium have allowed us to develop a novel paradigm for this preparation that includes: (1) paracellular fluid flow; (2) a crucial role for the junctions; (3) hypotonicity of the primary secretion; (4) an AQP role in regulation and not as a significant water pathway. These elements are remarkably similar to those proposed by the Hill laboratory for leaky epithelia.

Key words: Local osmosis — Permeability — Aquaporin — Knockout — Model

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Fluid Transport across Epithelia: a Historical Perspective

A very old idea to explain epithelial fluid transport by transcellular flow was pinocytosis. However, it has enjoyed a particularly bad name ever since Adrian Hogben's spectacular tirade against it (''the last refuge of the intellectually bankrupt'') in 1960 (Hogben, 1960). In fact, there is no convincing evidence for transcellular fluid transport by pinocytosis, and there are good arguments against that possibility (Reuss, 2000; Reuss, 2006).

In terms of their electrical resistance, epithelial layers differ: there are tight, intermediate, and leaky epithelia. This central fact was not recognized until the early 70's (Fromter & Diamond, 1972). After that time, it was observed that several epithelia that transport fluid isotonically tended to be leaky (Whittembury & Reuss, 1992). That was the case for those tissues in which most research was done: kidney proximal tubule, gall bladder, intestine, corneal endothelium (Whittembury & Reuss, 1992). However, there were apparent exceptions, such as salivary, lacrimal, or pancreatic glands, in which case there were two different classes of cells involved in the

generation of fluid, the acinar and the ductal ones. To determine the resistance of the different components was and remains challenging, given the geometries, so no clear picture has emerged on that item. There are also other fluid-transporting epithelia such as retinal pigment epithelium, choroid plexus, and ciliary epithelium which seemed to have intermediate to high resistance, but their geometry raised doubts about those values. Given those uncertainties, no clear picture has emerged yet as to whether fluid transport takes place only in leaky epithelia.

As another facet of the same discussion, for some epithelia such as kidney proximal tubule, gall bladder, and small intestine, debates arose between proponents of (a) a transcellular route for fluid transport, and (b) of a paracellular route. For arguments on both sides of this issue for kidney proximal tubule, cf. the review by Weinstein and Windhager (2001) and the paper by Tripathi and Boulpaep (1988). The first modern models for epithelial fluid transport were those of Peter Curran (Ogilvie, McIntosh & Curran, 1963; Katchalsky & Curran, 1965) and Jared Diamond (Diamond & Bossert, 1967); in both, fluid moved via the transcellular route, driven by local osmotic gradients at both the apical and basolateral cell membranes. These models began to appear in textbooks as explanations for this phenomenon.

When the matter was looked at more closely, however, problems were detected. A clear alarm was sounded by Adrian Hill (Hill, 1975a), who aptly described how fluid transported in the manner hypothesized by Diamond would have to be markedly hypertonic, while leaky epithelia transported isotonically.

Hill's objections brought the matter to a standstill. Still, if the flow could not well be transcellular, it had to be paracellular, and that also brought about problems. There were several papers from Hill's and other laboratories showing evidence interpreted to suggest solvent drag of solute that could only take place paracellularly. However, there were counterarguments that a similar drag of solute would take place if fluid would travel via lateral membranes and the paracellular space.

As a result, partly by inertia, partly because alternative explanations were difficult to prove, the local osmosis model not only survived but got entrenched in textbooks, which to this day predominantly explain fluid transport by some variant of local transcellular osmosis. The presence of water channels in fluid transporting epithelia has helped this thinking, as covered in what follows.

Water Channels

For water to traverse cell membranes, a question that arises is how does it cross the lipid bilayer. As the first water channel protein (aquaporin 1, or AQP1) was molecularly identified (Benga et al., 1986a,b; Denker et al., 1988; Preston & Agre, 1991; Preston et al., 1992), attention turned to its presence in epithelia. It was found that AQP1 was present in water-permeable segments of the kidney (Nielsen et al., 1993b), and in many secretory and reabsorptive epithelia (Nielsen et al., 1993a). This was consistent with fluid transport across epithelial cell membranes, and reinforced the hold of local osmosis on some reviewers of fluid transport (Fischbarg, 1995; Spring, 1998; Reuss, 2000).

Problems for Transcellular Water Transport...Again

Of course, the mere existence of aquaporins in the plasma membrane of a fluid transporting epithelial cell does not necessarily mean that water will use them as the route to traverse the layer. This has been brought home by findings in cases in which aquaporins were functionally inactive or absent. In the first category, humans with mutated non-functional AQP1 are phenotypically normal (Preston et al., 1994). To draw a definitive conclusion for this is difficult, as the absence of function could in theory be compensated by other systems. Still, one of the possible conclusions from that work is that AQP1 and the transcellular route are not essential for epithelial fluid transport.

Such findings could have been a mere call to attention in 1994. However, in the last decade the contributions of Alan Verkman's laboratory in collaboration with several other ones have been crucial to reopen the subject. They reported on work with aquaporin knockout mice (Verkman et al., 2000) and in many cases the results raised similar doubts as to whether aquaporins are the route of fluid transport through epithelia. For additional analysis of work with AQP knockout mice the reader is referred to a review by Hill's group (''What are aquaporins for?'' (Hill, Shachar-Hill & Shachar-Hill, 2004)). As a summation, we give a paragraph from it. In that context, SPH stands for ''simple permeability hypothesis'' according to which the role of AQPs is simply to increase the osmotic permeability of membranes above that of the bilayer: ''Observations on such knockouts and mutants have shown a wide range of effects on fluid transfer rates. SPH would predict that the removal of a major pathway of water flux across membranes would have drastic effects on biological function at the cellular, tissue and whole animal level. However, these have been remarkably few, and those effects that have been observed are hard to interpret by the SPH...''

As an illustration, we will discuss below the evidence for corneal endothelium (Kuang et al., 2004), in which deletion of AQP1 decreased osmotic permeability by ≈40%, but had no significant effect on fluid transport. One more striking example for these

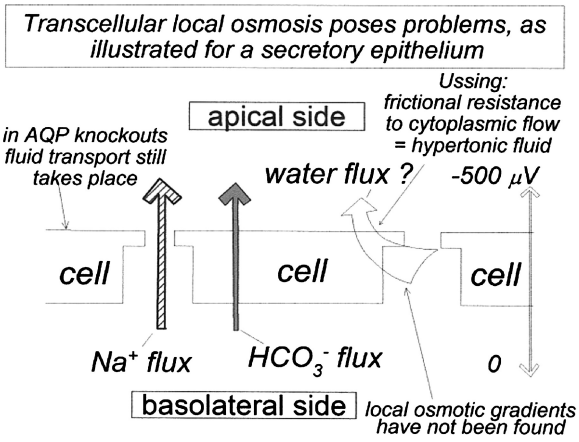

Fig. 1. A schematic summary of problems encountered attempting to explain fluid secretion by transcellular local osmosis. The ionic fluxes indicated are those found in corneal endothelium. More detail is given in the text.

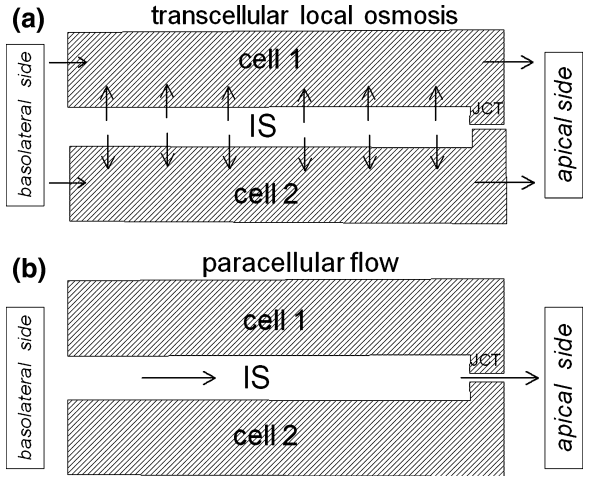

Fig. 2. Schematic diagrams of the intercellular spaces (*IS*) and the leaky tight junctions (*JCT*) between two adjoining cells of a secretory fluid-transporting epithelial layer. The arrows indicate the direction and route of the hypothesized water movements, transcellular (*top*) and paracellular (*bottom*).

trends has been published recently (Oshio et al., 2005). In that work, the osmotic permeability of choroid plexus and rate of cerebrospinal fluid production were determined in normal and AQP1 null mice; AQP1 deletion reduced osmotic permeability by ≈80%, but reduced CSF production by only ≈25%.

Figure 1 summarizes schematically the main objections raised this far against the transcellular local osmosis explanation for fluid transport. The fact that fluid transport is affected little by the absence of water channels has been dealt with above. To that one needs to add the absence of widespread evidence for local concentration gradients. Last but not least, there is Ussing's objection (Ussing & Eskesen, 1989): he showed that given transcellular flow of water and solute, the friction of water with the cytoplasm fixed elements will mean that the fluid transported will always be hypertonic. From this, transcellular flow could not account for the isotonic fluid transport seen across leaky epithelia.

Discriminating between Paracellular and Transcellular Routes

Given these two possible pathways for water across an epithelial layer (Fig. 2), there are ways to distinguish between them. Since 1978 onwards there has been a steady succession of papers describing evidence for paracellular water flow. (For a review *see* Whittembury & Reuss, 1992). More recently, evidence for paracellular flow has been reviewed by Shachar-Hill and Hill (2002). They describe studies using paracellular probes in gallbladder, salivary gland, intestine, and Malpighian tubule; the fraction of fluid traversing the paracellular path, including the junction, is near 1 in all these cases. They propose that fluid flow is generated at the junctions by a non-osmotic mechanism. To that list we add the corneal endothelium, for which our own evidence (discussed below, Sanchez et al., 2002) suggests that fluid transport also traverses the paracellular pathway, including crucially the junctions.

Evidence against paracellular flow appeared in a report finding no water flow across the junctions of cultured MDCK epithelial layers (Kovbasnjuk et al., 1998) as determined using a complex optical-computational technique. However, such layers are known to have very poor fluid-transporting ability (Timbs & Spring, 1996), so that observation (Kovbasnjuk et al., 1998) may not have relevance for epithelia transporting fluid in normal amounts. Hence, in balance, the preponderance of evidence this far suggests that the flow is paracellular in the leaky layers above.

Solute-Solvent Coupling in the Tight Junction: the Electro-osmosis Paradigm

Electro-osmosis was first suggested by Hill in 1975 (Hill, 1975b) as a possible mechanism for the solute-solvent coupling underlying fluid transport across epithelia modeled as a double membrane system. It was also cited as or can be an explanation of the evidence obtained in several experimental papers: Spring and Paganelli (1972) in *Necturus* kidney proximal tubule (although they interpreted current-induced fluid movements as due to concentration changes); Naftalin and Tripathi (1985) in rabbit ileum; Lyslo et al. (1985) in corneal endothelium; Ussing and Eskesen (1989) in frog skin glands;

Nielsen (1990) in frog skin glands; Hemlin (1995), in rat jejunal epithelium; Sanchez et al. (2002) in corneal endothelium. In addition, also for kidney proximal tubule, electro-osmosis along the paracellular spaces was analyzed and found insufficient to account for the observed rate of fluid transport (McLaughlin & Mathias, 1985). In spite of this body of evidence, acceptance of a possible role for electro-osmosis in Biology has been hampered. Theory, experimental work and industrial applications of electro-osmosis are well developed, but application to Biology is complex. For instance, the classical Helmoltz-Smoluchowski treatment developed for ideal capillaries and physico-chemical membranes cannot be applied directly, as explained below and elsewhere (Fischbarg, Rubashkin & Iserovich, 2004; Rubashkin et al., 2005). In addition, the role of the tight junctions in fluid-transporting leaky epithelia seems to have escaped prior attention.

For a number of these reasons, electro-osmosis is still not widely accepted. For instance, in recent reviews (Reuss, 2000, 2006) it is dismissed on the weight of two papers: (1) Wedner and Diamond (1969), and (2) Van Os et al. (Van Os, Michels & Slegers, 1976). However, from our own recent evidence, the impact from these two papers needs to be updated.

According to Wedner and Diamond, the absorbate generated by transepithelial voltages across mammalian gall bladder was very hypertonic (coupling ratio Jv/Js was 0.5 to 1 liter/osmol, short of the ideal isotonic coupling of 3.3 liters/osmol). In our case, years ago we obtained similar results (Kuang, Cragoe & Fischbarg, 1993) working with rabbit corneal endothelia mounted in tightly clamped chambers used to determine fluid transport with the Bourguet technique. We observed electro-osmotic fluid movements (in both directions), with the currents required to generate them being 4 times larger than required for ideal isotonic coupling. That far, we were in qualitative agreement with the results of Wedner and Diamond. However, more recently, we have reexamined the question using the same rabbit corneal endothelial preparations but a different mounting procedure, that of Dikstein and Maurice (1972), which minimizes edge damage. Dramatically, in this case the current and fluid movements were coupled isotonically (Sanchez et al., 2002). The overall coupling ratio reported there (Sanchez et al., 2002) was $r = 2.37$ (μm/h) (cm^2/μA) = 0.066 cm^3/coul, from which $r \times F/2 = 3.2$ liters/osmol (F = Faraday's constant). Hence, in that system, electro-osmosis can account for isotonic fluid transport.

As for the paper of Van Os et al. (1976), it raises the possibility that apparent electro-osmotic fluid movements could be due to current-induced changes in salt concentrations in unstirred layers. However, such concentration changes require a build-up time, whereas electro-osmotic movements are bound to be instantaneous, as discussed by McLaughlin and Mathias (1985). In one instance, rapid development of electro-osmotic fluid flow was reported (Naftalin & Tripathi, 1985). In this connection, we had described earlier (Kuang et al., 1993) that fluid movements across corneal endothelium responded instantly to changes in current. More recently, we have documented that the fluid movement response to a current pulse takes place in 1 s (Fig. 7 in Sanchez et al., 2002), which is an interval too short for salt buildup (Fig. 9 in Sanchez et al., 2002), therefore suggesting electro-osmosis (Sanchez et al., 2002).

In view of this, we propose that electro-osmosis be considered without prejudice as a possible explanation for epithelial fluid transport.

Recent Evidence from Our Laboratory. I. Paracellular Electro-osmotic Fluid Flow with Junctional Coupling

The experimental part of this work has been published (Sanchez et al., 2002) and reviewed (Fischbarg, 2003). We have also generated a novel theoretical treatment of electro-osmotic coupling in the tight junctions (Fischbarg et al., 2004; Rubashkin et al., 2005). Here we will highlight some of the main points involved.

A Better Technique?

Schematic diagrams of the intercellular spaces (IS) and the leaky tight junctions (JCT) between two adjoining cells of a secretory fluid-transporting epithelial layer are shown in Fig. 3. The arrows indicate the direction and route of the hypothesized water movements, transcellular (*top*) and paracellular (*bottom*).

Figure 4 shows the Dikstein-Maurice chamber utilized. As remarked above and shown in what follows, with this technique the calculated coupling was near-isotonic, whereas with another technique for fluid transport measurements (Fischbarg, Lim & Bourguet, 1977) the coupling was hypertonic (~4× more electrical current was needed to generate similar fluid flows, cf. Kuang et al., 1993). To be noted, in the Dikstein-Maurice procedure, the clamping force is less, and the corneal stroma remains in its normal, unswollen condition. These or other undetermined factors may account for the improved coupling observed. To be noted, in a normal-thickness cornea in the steady state, the endothelial fluid transport is virtual, as the tendency of the pump to drive fluid from stroma to aqueous is equal and opposite to the tendency of fluid to leak back into the stroma driven by the imbibition pressure of the stromal

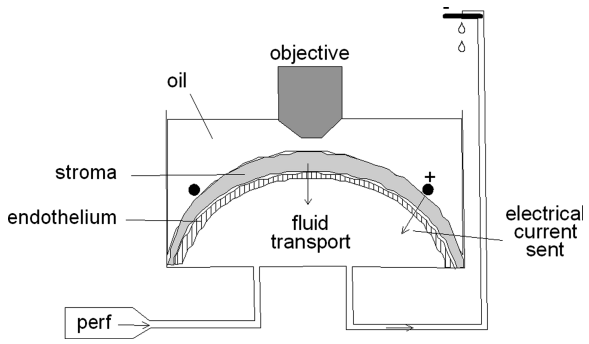

Fig. 3. Transendothelial fluid transport measurement by the Dikstein-Maurice technique. De-epithelialized rabbit corneas (*in vitro* endothelial preparations) are placed in a perfusion chamber (T = 36.5°C), and fluid movements are detected as stromal thickness changes. Electrical current is passed through the electrodes shown.

mucopolysaccharides. Vectorial fluid and electrolyte transport rates are determined in preparations in which the corneal stroma is swollen, which eliminates the stromal imbibition pressure.

Paracellular Electro-osmosis. Experimental Results

Figure 5 shows the main results obtained. In preparations originally in steady state (pump = leak, stromal thickness remains constant), sending electrical currents across the preparation disturbs the steady state and results in net fluid movements across the endothelium. The fluid movements are of magnitude and direction corresponding to the currents. A control panel shows that in the absence of an intact corneal endothelial cell layer, no such electro-osmotic coupling occurs. From other control experiments (Sanchez et al., 2002; *not shown* here), integrity of the intercellular leaky tight junctions is required for electro-osmotic fluid flow.

Osmolarity of the Secretion

In our current understanding, electrical mobile charges traversing the junction will develop electro-osmotic coupling and will convect fluid across the junction. In principle, the fluid being convected will assume the composition it had inside the junction. Due to the effects of the macromolecules inside the junction, in our estimate (Rubashkin et al., 2005) the osmolarity of that fluid would be ~0.7 of that in free solution. Of course, upon emergence, this hypotonic secretion would undergo osmotic equilibration via membrane AQPs, perhaps in cyclic fashion. This estimate is consistent with a hypothesis for junctional flow generating hypotonic fluid (Shachar-Hill & Hill, 2002).

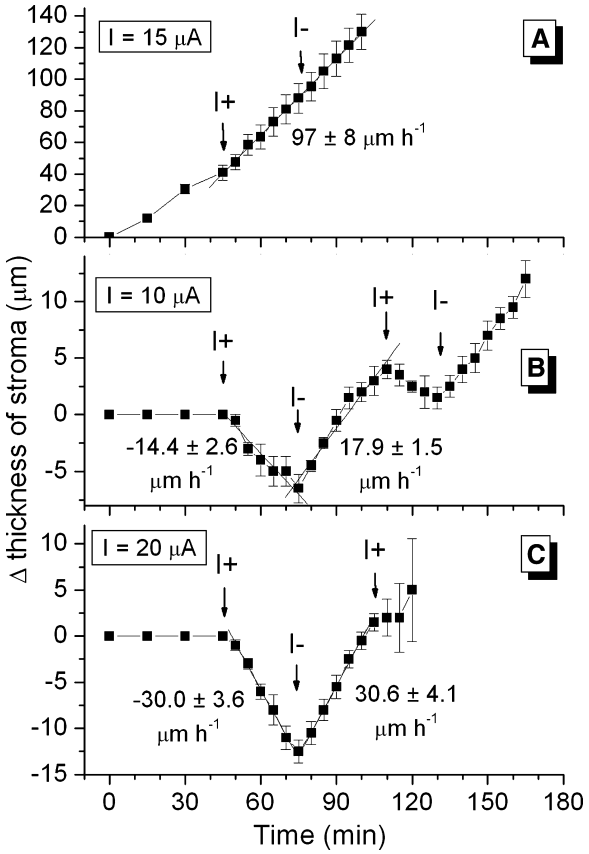

Fig. 4. Externally applied electrical currents ($I+$, $I-$) generate transendothelial fluid movements detected by stromal thickness changes. During the control period ($0 < t < 45$ min), the thickness of the stroma remains constant, as the corneal endothelial (virtual) rate of fluid transport is exactly balanced by the stromal imbibition (negative) pressure. When external currents are applied, as fluid leaves the stroma across the endothelium, stromal thickness decreases, and vice versa. Numbers by the fitted lines denote the values of the slopes.

Selectivity of the Junction

For fluid to move in the same direction of the current by junction-coupled paracellular electro-osmosis (Fig. 5 and Fig. 1 here; Fig. 4 in Sanchez et al., 2002), the junction has to have fixed negative charges, resulting in some cationic selectivity for which there is indeed evidence (Lim, Liebovitch & Fischbarg, 1983). This poses the question of whether such charges can be experimentally manipulated. We have examined this with the experiments shown in Fig. 6. As can be seen there, the same current I^+ is applied to a standard preparation and to another one treated with the polycation polylysine. The direction of fluid flow is the usual one in the untreated preparation (*bottom*), but reverses in the polylysine-treated one (*top*). This observation is consistent with paracellular, junction-coupled electro-osmotic flow, with the polylysine agent causing a reversal of the junctional

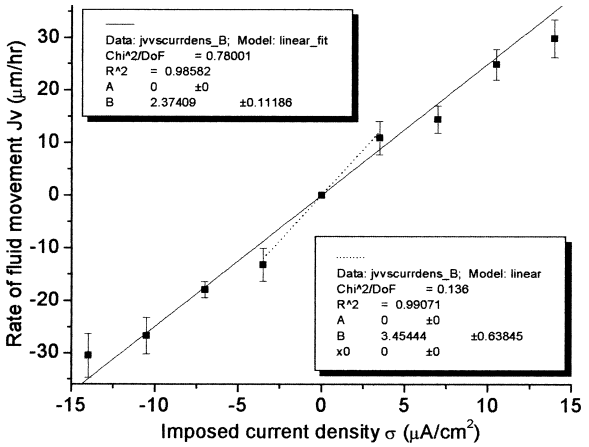

Fig. 5. Fluid movements as a function of the externally imposed current. Solid line: linear fit for all the points. Dotted line: linear fit for small currents.

charge and hence a reversal of the direction of current-induced fluid flow.

Paracellular Electro-osmosis. Theory

We have begun to examine the physico-chemical mechanisms that might be at play in the paracellular pathway. Within it, we have concentrated our attention on the leaky tight junctions (TJ); they are the site of a very high voltage drop and therefore the likeliest place for paracellular electro-osmotic coupling. Recent developments on tight junction physiology (Van Itallie & Anderson, 2004) have provided the necessary background. To be noted, in all prior efforts, electro-osmosis in the TJs seems to have been disregarded. Our treatment takes into account that leaky tight junctions (TJ) possess a significant amount of protruding macromolecules, some of them electrically charged. This results in a partition coefficient which is smaller for electrolytes in the TJ compared to free solution. This in turn could be a basis for hypotonic secretion, as convection will carry fluid at the TJ local concentration. The model embodies systems of electro-hydrodynamic equations for the sterically restricted or strand regions of the TJ, namely the Brinkman equation (an expression that describes water flow across polymeric materials) and the Poisson-Boltzmann differential equation. We obtain analytical solutions for a system of these two equations, and are able to derive expressions for the fluid velocity profile and the electrostatic potential. We arrive at a modified version of the Helmholtz-Smoluchowski expression. We illustrate the model by employing geometrical parameters and experimental data from the corneal endothelium.

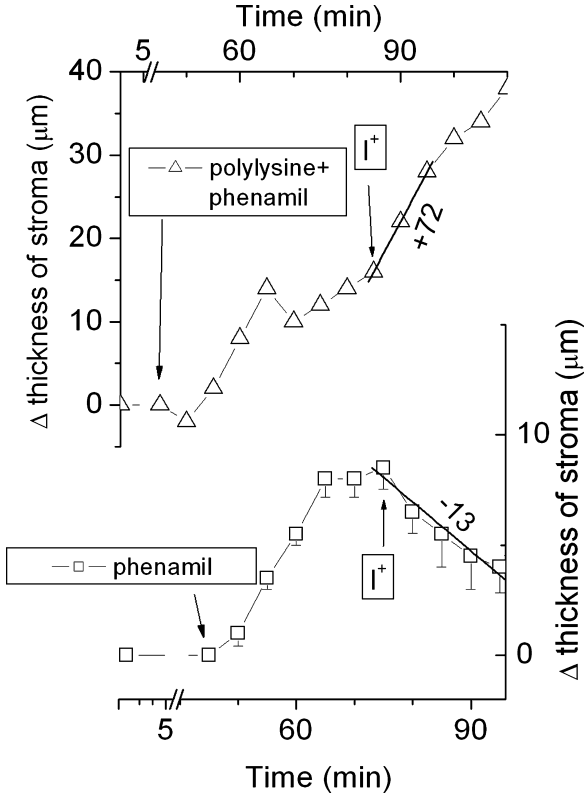

Fig. 6. Rabbit corneal endothelial preparations. Current-induced fluid flow by a current of +15 μA, in the presence (*top*) and absence (*bottom*) of polylysine (MW 100, 4 mg/ml). Phenamil (1 μM) was present in both cases to exclude Na^+ recirculation.

Recent Evidence from Our Laboratory. II. Fluid Transport across AQP1 Null Mouse Corneal Endothelium

A paper with our detailed findings has been recently published (Kuang et al., 2004). As in the prior paragraphs, we comment on some relevant highlights.

As we reported, Figs. 7, 8 and 10 show that the values of the rates of fluid transport by both wild type and AQP1 null layers fall at or near a range usual for these preparations (4.0 ± 0.5 μl h^{-1} cm^{-2}; Narula et al., 1992). As Fig. 7 shows, the records from each type look very much alike, both in amplitude and time course. Figure 8 shows the values of all the successful experiments (9 with each type of cell) against the date in which they were performed. No particular trend seems discernible, except that the rates from both groups show great overlap. The averages were 4.3 ± 0.6 for the wild type and 3.5 ± 0.6 for the AQP1 null. The average rate for the wild type was therefore slightly (19%) larger, but with standard statistical handling, that difference was not significant. If one perseveres in the analysis, as shown also in Fig. 8, the Lorentz fits to the distributions of

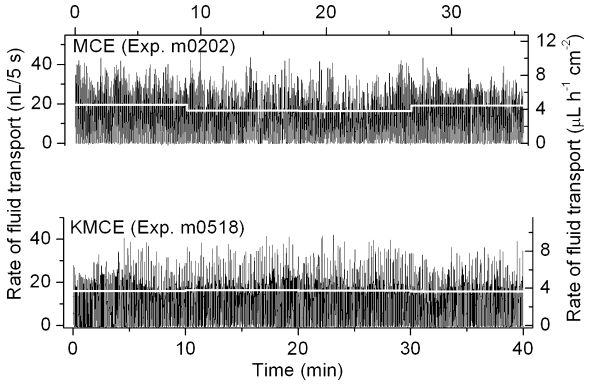

Fig. 7. Representative records of experiments in which we monitored spontaneous fluid transport by wildtype (*top panel*) and AQP1 null mice (*bottom panel*) corneal endothelial cell layers grown on permeable inserts. Vertical deflections represent volume accumulated during the 5 second recording intervals used. Every 5 s, the accumulator is reset and the process repeated. The upward direction (positive y coordinate) corresponds to fluid movement from the bottom half-chamber (basolateral side) to the top one (apical side), the normal direction of fluid transport in this preparation. The fluid level in the top chamber is 3 cm above that in the capillary sensor communicating with the bottom chamber. That pressure keeps the insert membrane steady against its support and ensures that any positive fluid displacement represents active transport performed against a pressure head. T = 37°C. The white lines denote rate of fluid transport values averaged for 10 minute intervals.

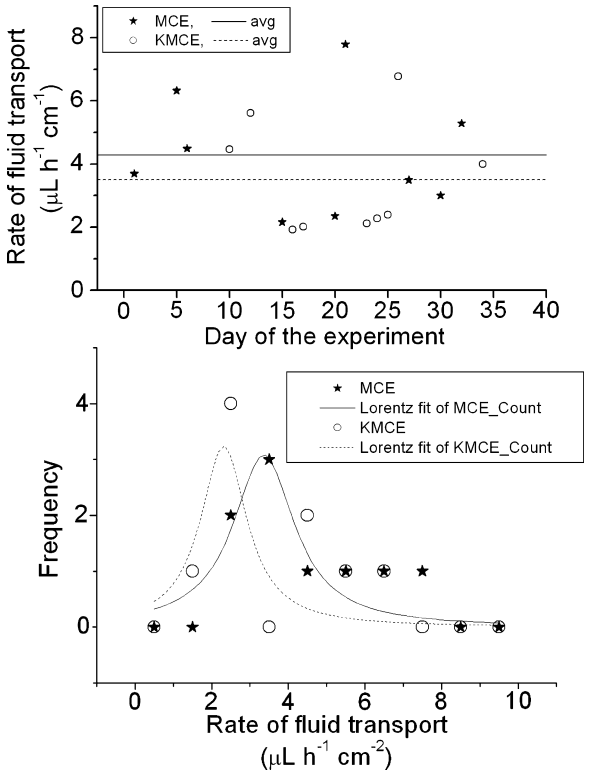

Fig. 8. *Top*: Results obtained with both MCE and KMCE appear against the correlative days in which experiments were performed. The lines correspond to the average values for both groups of experiments (solid: MCE, 4.3; dashed: KMCE, 3.5). *Bottom*: Incidence of rates for both types of cells. Rates were distributed in bins (1 μl h^{-1} cm^{-2} wide). Their frequency distributions were then fit to the Lorentz functions indicated.

the rates observed yield peaks, with the one for AQP1 null cells falling at a value 1 μl h^{-1} cm^{-2} smaller than that for wild-type cells. However, the overlap between both distributions is still very considerable.

In balance, it is conceivable that with more experiments a slight deficit in the AQP1 null cells could be made more apparent. Still, the fact that after 9 experiments that is not the case indicates that any such difference is bound to be small and the overlap will dominate.

A legitimate question in this connection is whether other AQPs could be present in the tissue and be compensating for AQP1 absence. The answer is twofold:

(1). We have looked in corneal endothelium for the presence of mRNA encoding all AQPs found in the eye (AQP 1–5). We found only AQP1 (Wen et al., 2001); a prior study had reached similar conclusions (Hamann et al., 1998).

(2). The deletion of AQP1 has a very noticeable affect on the endothelial osmotic permeability; the P_f value for the wild-type cells is 74 ± 4 μm s^{-1}, and that of the AQP1 null cells is 44 ± 4 μm s^{-1}. From this, AQP1 accounts for ~ 40% of the cell P_f in the wild type, and the decrease in P_f in the AQP1 null cells has not been compensated (Fig. 9, Fig. 10).

From the above, the dichotomy observed in other AQP knockout systems (Hill et al., 2004) is present here as well: while P_f goes down by 40%, fluid transport decreases by perhaps 19% (Figure 10). Once more, this militates against an explanation of fluid transport involving transcellular local osmosis. A more extensive discussion of transcellular local osmosis is offered below under "Problems with Apical Osmosis".

A Role for AQP1 in Endothelial Fluid Transport?

As Figs. 9 and 10 also show, unexpectedly, volume regulatory decrease is hindered in the AQP1 null mice (by some 40%). In this connection, as we have argued (Kuang et al., 2004), there is evidence that in the endolymph-producing dark cells in the inner ear epithelium, PDZ domain–containing proteins that bind the C termini of EphB2 and B-ephrins can also recognize the cytoplasmic tails of anion exchangers and aquaporins (Cowan et al., 2000). It is therefore conceivable that AQPs and volume-sensitive transporters might co-localize in such a way that lack of AQPs might adversely affect the function or the

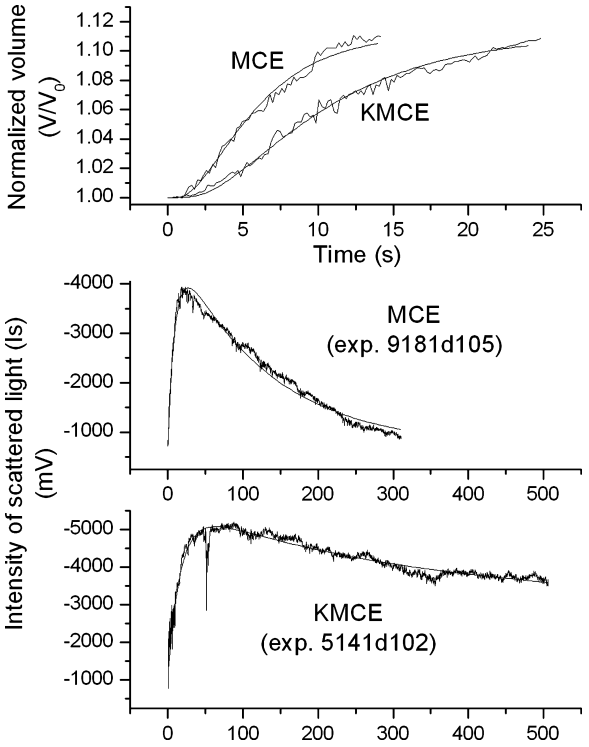

Fig. 9. *Top panel*: the expanded time scale depicts the initial or "osmometric" phase of the volume changes in the other two panels. *Middle* and *bottom panels*: recordings of the time course of the cell volume of plated corneal endothelial cells of wild-type and AQP1 null mice after 10% hypo-osmotic challenge as determined by light-scattering. T = 37°C. Experiment # 9181d105 (middle panel), representative of 19, exemplifies how in wild-type cells the volume recovery was complete. Experiment # 5141d102 (*bottom panel*), representative of 11, shows that during a time interval almost twice that of the top panel, the recovery was incomplete.

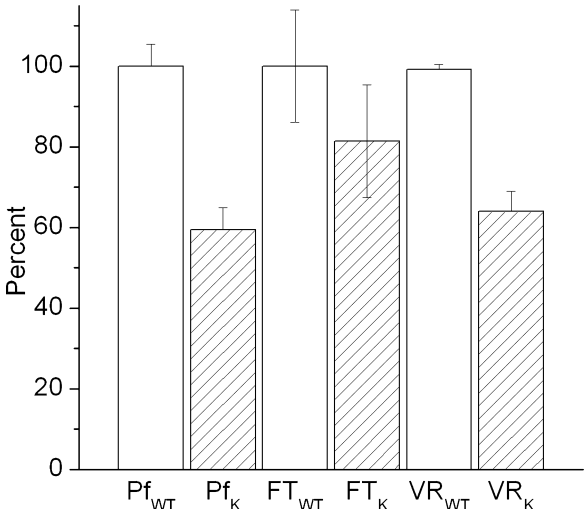

Fig. 10. Comparison of averages for: (1) cell membrane osmotic permeability (*Pf*), (2) rate of fluid transport (*FT*), and (3) extent of regulatory volume decrease (*VR*) values for wild-type mice (*WT*) and AQP1 null mice cells (*K*). For (1) and (3), values were determined in cells grown on glass coverslips after a 10% hypotonic challenge. For (2), experiments were done with cell layers grown on permeable supports.

expression of such transporters in plasma membrane domains. Regulatory roles for AQPs have been suggested, e.g., for cell volume set point (Stamer et al., 2001), cell homeostasis (Patil et al., 2001), or osmosensing (Hill et al., 2004), so it may be fitting to see AQP1 connected here once more to volume regulation. As mentioned above, according to estimates (Rubashkin et al., 2005; Shachar-Hill & Hill, 2002), the fluid moving through the junctions may emerge hypotonic. That would lead to secondary osmotic equilibration through AQPs. If that equilibration involves cycles of volume regulation, that would explain why deletion of AQPs has more pronounced effects in those epithelia that generate relatively larger amounts of fluid (Hill et al., 2004).

Additional reasons for why AQPs do not seem to be connected to substantial transcellular water flows are given in what follows.

Problems with Apical Osmosis

Given the results with the AQP1 null mice, advocates of transcellular local osmosis have argued that even with a 40% decrease in P_f, fluid transport could still proceed if the local osmotic gradient would just increase so as to drive the same amount of fluid as previously. This "gradient increase" proposal has, however, a glaring flaw. Transcellular local osmosis requires two gradients, one between intercellular spaces and the cell, another one between the cell and the apical compartment. The simplest for this argument is to concentrate on the limitations inherent to the hypothetical gradient at the apical membrane.

The corneal endothelium has an important advantage: it is a monolayer of remarkably flat cells, with an absence of extensive apical infoldings or villi. This allows one to model the events at the apical cell membrane using a hypothetical flat cell membrane and one-dimensional convection-diffusion equations. The geometry considered is given schematically in Fig. 11.

One tenet of local osmosis is that a local osmotic gradient will exist at this membrane. If the gradient forms outside the cell, the concentration profile will look as depicted in Fig. 11, rising immediately outside the membrane as electrolyte transport systems lead to accumulation of salt in there, and dissipating along the unstirred layer (of width $\Delta x = 50$ μm) clinging to the cell.

To introduce the reader to the inherent difficulties, let us consider salt convection. The rate of translayer bicarbonate transport by corneal endothelium is $Js_{exp} \sim 0.5 - 0.7$ μEq h^{-1} cm^{-2} (Diecke et al., 2004; Hodson & Miller, 1976); that would contribute to accumulate salt right outside the

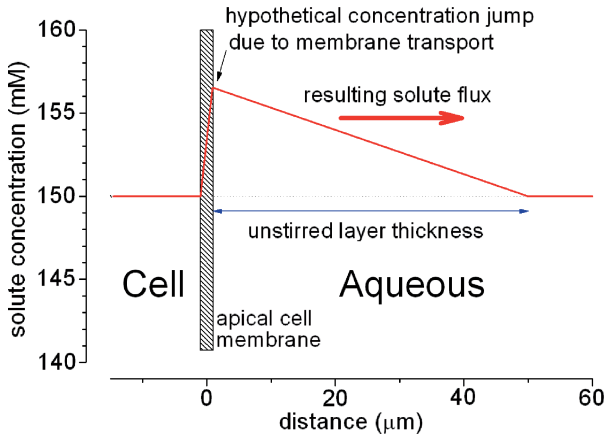

Fig. 11. Schematic depiction of a hypothetical solute concentration profile at the apical membrane boundary of corneal endothelial cells. To the left of the membrane, the cell cytoplasm. To the right, an unstirred layer clinging to the cell followed by the bulk of the bathing medium (aqueous humor *in vivo*).

membrane. On the other hand, fluid transport by that layer is in the order of $Jv = 4.5$ μl h^{-1} cm^{-2} (Fischbarg et al., 1977). Fluid emerging from the apical cell membrane at that rate will generate a convection flux *Jsc* of the order of:

$$Jsc = Jv \times C\mathrm{iso}/2,$$

where *C*iso is isotonic concentration of particles, or 300 mOsmoles/l (we assume that the concentration near the membrane has not changed much). However, from this calculation, $Jsc = 0.7$ μEq h^{-1} cm^{-2} $\approx Js_{exp}$. Hence, by this reasoning, the salt transported by the membrane (Js_{exp}) is being convected away immediately, with no salt (or osmotic) gradient forming at the boundary.

Ignoring convection does not bring relief either. Using the value for the osmotic permeability above for the wild-type mouse endothelial cell membrane ($Pf_{wt} = 74$ μm s^{-1}), the concentration gradient ΔC_{wt} (of monovalent ions) that would have to exist across the apical membrane to drive the experimental flow Jv_{wt} observed (4.3 μl h^{-1} cm^{-2}) is:

$$\Delta C_{wt} = Jv_{wt}/(2 \times Pf_{wt} \times V_w) = 4.5\,\mathrm{mmol/l}$$

However, such gradient would result in a diffusional flow Jsd_{wt} across the unstirred layer (from the membrane boundary to the bulk solution, left to right in Fig. 11) of:

$$Jsd_{wt} = D \times \Delta C_{wt}/(2\Delta x) = 48.4\,\mu\mathrm{Eq\,h^{-1}cm^{-2}}$$

Such flow would be 69 times larger than the experimentally determined bicarbonate transport *Js*, which grossly violates the First Law of Thermodynamics. Since the corneal endothelial *Pf* of the knockout mice is even less (44 μm s^{-1}), for that case the hypothetical gradient necessary would be larger and the calculated flow across the unstirred layer would be 95 times larger than the experimental value. In fact, the only gradient that is compatible with the flux and unstirred layer values given is ~0.07 mmol/l.

These limitations of apical local osmosis have appeared before in the literature. In a review from the Hill group (Shachar-Hill & Hill, 2002), there is a comment that such process would violate the convection-diffusion equation. Our own laboratory examined the issue of apical osmosis already in 1985 (Fischbarg, Liebovitch & Koniarek, 1985). We wrote then: "Much as in other models, we confirm that only rather unrealistically high values of the cell membrane permeability lead to isotonic transport. We have also found, however, that isotonic transport can occur at much lower values of the cell membrane permeability if the concentration within the cell differs slightly from that in the ambient medium." In other words, apical transcellular local osmosis might operate if the cell is made somewhat hypotonic. There are, however, problems with that possibility as well. Liebovitch and Weinbaum (1981) examined a model based on that assumption (lateral spaces definitely hypotonic, cell less so but still hypotonic). One resulting complication was that cells exported fluid in two opposite directions, since both the apical and basal compartments were hypertonic to them. The other complication ensues from the value one would have to assume for the cell hypotonicity (perhaps 1% or 1.5 mmoles/l). As we examine elsewhere (Diecke, Zhu, Kuang, and Fischbarg, *in preparation*), that osmolarity displacement would be enough to trigger cell volume regulation, which in turn would diminish or nullify the original gradient. In view of this, salvaging local osmosis would now perhaps require an oscillatory mechanism. A cyclic process has been hypothesized for transcellular osmosis (Fischbarg, 1997), but findings after that militate against it, as in the endothelium the same AQP1 isoform would have to be modulated independently in both the apical and basolateral membranes, which seems very unlikely. Oscillations connected with control of the osmolarity of fluid transported have been hypothesized (Shachar-Hill & Hill, 2002). In this connection, we called attention to our evidence that the primary secretion by corneal endothelium could be hypotonic (Sanchez et al., 2002; Fischbarg, 2003), which carries implicitly that the regulatory mechanisms of the cell would react to such apical hypotonicity. Still, these regulatory oscillations would control paracellular rather than transcellular fluid flows, which does not help the cause of transcellular local osmosis.

The consideration of apical local osmosis is crucial to judge local osmosis. The limitations given here may be simple and obvious, but somehow they have apparently not been detailed before. Perhaps these

and other arguments will now help prompt readers to ask as we do: "precisely what is the experimental evidence for transcellular local osmosis?"

Recent evidence from Our Laboratory. III. A Corneal Endothelial Model Points to Paracellular Flow and Electro-osmosis

There are a few variants of modern mathematical models to describe epithelial transport and electrophysiology (Lew, Ferreira & Moura, 1979; Latta, Clausen & Moore, 1984; Novotny & Jakobsson, 1996). To model the corneal endothelium, for convenience we have adopted the methods originated by Leon Moore and colleagues (Latta et al., 1984), as applied by Verkman and colleagues (Hartmann & Verkman, 1990; Verkman & Alpern, 1987). Their algorithm was suitably modified by us for computational reasons and on the basis of what is known about the presence and distribution of transport proteins in the corneal endothelial membranes. The structure of the program and our results with it are reported in a recent paper (Fischbarg & Diecke, 2005). Another technical achievement that may prove useful was that we started from five known parameter values and were able to build a table of eleven parameters using conservation equations. As reported (Fischbarg & Diecke, 2005), the model has the complexity required (Fig. 12) to reproduce in detail experimental findings, including the characteristic low electrical potential difference across this preparation and its dependence on ambient factors (Fig. 13).

The model is to us very illuminating. It paints the endothelial cells as devoted not to the net transport of salt (they transport predominantly anions) but instead to the generation of an intense recirculating local current (Fischbarg & Diecke, 2005). In fact, it was the insight emerging from the model what led us to revisit the issue of paracellular electro-osmotic water flows in 2000–2001, leading to the results published shortly afterwards (Sanchez et al., 2002).

As a sample of the model capabilities, we show here (Fig. 13) how well it accounts for the effect of ambient [Na^+] on transendothelial potential difference. Why it should be so is difficult to express intuitively, but the saturation curve in that figure may have to do with the binding constants for Na^+ at sodium-bicarbonate cotransporter binding sites.

The model also predicts rates of fluid transport by the cell layer. It does so in two different modes:

(1). local osmotic mode, in which water movement is given by: $Jv = 2\ Js_{calc}/Ciso$, where Js_{calc} is the total net monovalent salt transport rate (from stroma to aqueous) calculated by the program.

(2). paracellular electro-osmotic mode, in which $Jv = Ioc \times r$, where Ioc is the calculated local open-circuit current density circulating around the cells (the circuit is given in Fig. 13; to be noted, it includes the paracellular pathway) and r is a coupling ratio experimentally determined between current and fluid movements for rabbit corneal endothelium (Sanchez et al., 2002).

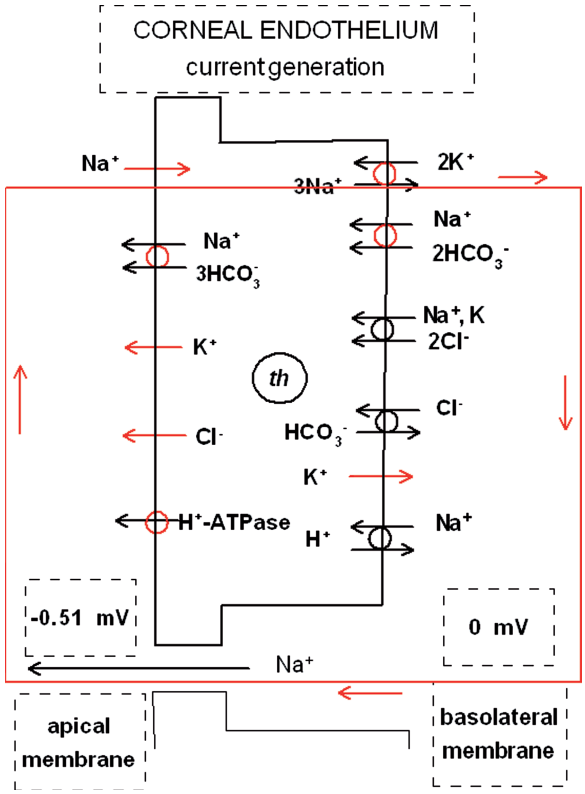

Fig. 12. Tangential section across idealized corneal endothelial cells. The illustration lists all the known transporters and channels that in our model (Fischbarg & Diecke, 2005) contribute to either cell homeostasis or translayer transport. Most locations are well known; some are presumed based on evidence. The ions transported are identified; *th* represents a source for H^+. Heavier arrows denote electrogenic elements which contribute to the local recirculating open circuit current, shown as a thin rectangle.

The predictions of the program are compared with the experimental results obtained when the preparations are bathed in bicarbonate-free medium. Under those conditions, transport of bicarbonate ceases, and for all practical purposes so does transport of salt. On the other hand, about half of the *Ioc* remains, in all likelihood carried mostly by the lateral Na^+ pumps, apical ENaCs and other apical Na^+ pathways. As can be seen in Fig. 13, the predictions agree with paracellular electro-osmosis and not with local osmosis, which instead predicts that as salt transport ceases, so should water movement. Years ago, Doughty and Maurice (1988) reported that the corneal endothelium could transport fluid in the absence

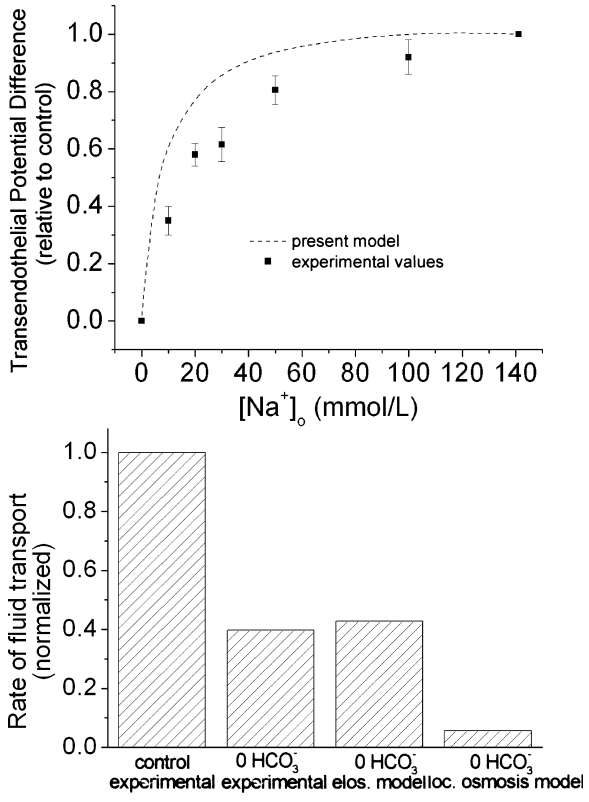

Fig. 13. *Top*: Transendothelial electrical potential difference as a function of the ambient sodium concentration. The predictions of the model are compared to experimental data reproduced from (Fischbarg & Lim, 1974). *Bottom*: Rate of fluid transport across corneal endothelium. The effect of replacing all ambient HCO_3^- by Cl^-, as reported in Kuang et al., (1990), is compared to predictions from the two indicated versions of the model.

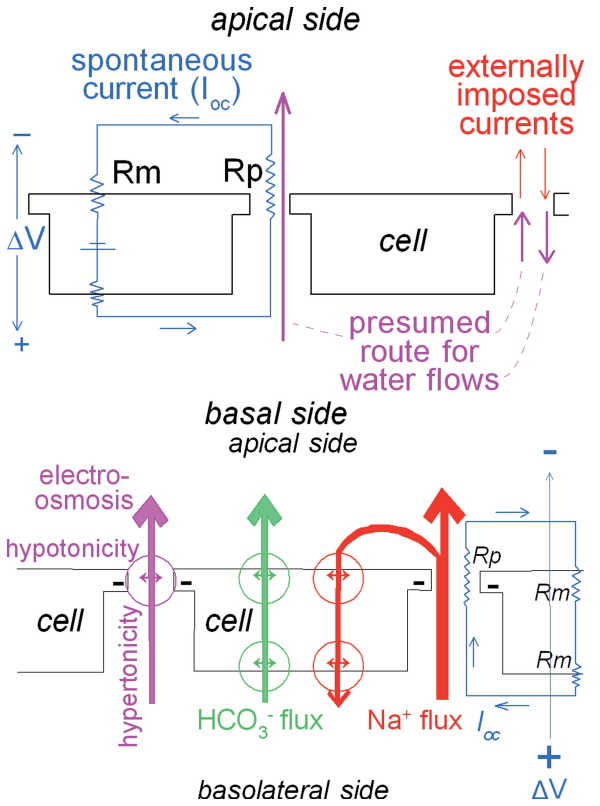

Fig. 14. Diagrams showing elements of the mechanism postulated for fluid transport across corneal endothelium. *Top*: Left side, spontaneous electrical current through corneal endothelium driven by the existing potential difference ΔV. *Ioc*: open-circuit current; *Rm*, *Rp*: specific resistances of the membrane and paracellular pathways, respectively. Right side: since $Rm >> Rp$, externally imposed currents traverse almost exclusively the paracelular pathway. Water flows through the paracellular pathway, coupled to the cation currents either spontaneous (*left*) or imposed (*right*). *Bottom*: The separate pathways for the translayer ionic fluxes of HCO_3^- through the cell, and of Na^+ through the paracellular; part of this last flux will be recirculated through the cell. Circles denote putative regulation sites. As junctional coupling is hypotonic, fluid production creates an osmotic imbalance across the junction that cell regulatory mechanisms will tend to correct, perhaps in cyclic fashion.

of bicarbonate. That finding was confirmed but attributed to residual endogenous (Kuang et al., 1990) or exogenous (Bonanno, 1994) bicarbonate. The model now allows one to picture events more clearly: the residual fluid transport is truly independent of bicarbonate (or net vectorial transport of electrolytes), and can only occur by a non-local osmotic mechanism, such as paracellular junction-coupled electro-osmosis. The model we propose is therefore consistent with the rest of the evidence and provides further support for paracellular non-local-osmotic fluid transport.

The Emerging Model for Fluid Transport in Corneal Endothelium

The elements of the model we propose for corneal endothelial fluid transport are shown in Fig. 12 and Fig. 14. Those figures show how the electrogenic transporters and channels contribute to generate an electrical potential difference and a resulting electrical current recirculating around the cells. Since the junction is "leaky tight", with a specific resistance for the layer of $\sim$ 20 ohm cm^{-2}, the current is relatively intense; for the cross-sectional area of the junctions, we calculate a current density of $\sim$ 60 mA cm^{-2}. This paracellular current is carried mostly by Na^+, as Fig. 14 shows. As the junctions are cation-selective, the current generates fluid movement by electro-osmotic coupling. The coupling results in somewhat (perhaps 25%) hypotonic emerging fluid. This entails that the fluid left behind at the intercellular spaces is correspondingly hypertonic. Such osmolarity difference is bound to be sensed by the cell and to trigger cellular mechanisms that will affect the postulated sites for regulation at basolateral and apical sites for HCO_3^- and Na^+ transports, and at the junction to

modify the characteristics of the coupling. It is conceivable that such regulation might take place in cyclic fashion. There may be a role for AQP1 in this regulation, which might explain the effects seen on fluid transport in this and other preparations in experiments done with AQP1 null cells. This view is consistent with a characteristic of those results noted by Verkman and colleagues, namely, that effects of AQP deletion are more pronounced in epithelia that generate higher rates of fluid transport. Thus, AQP deletion reduced near-isosmolar fluid transport in kidney proximal tubule (Schnermann et al., 1998) and salivary gland (Ma et al., 1999), where fluid transport is rapid, but not in lung (Bai et al., 1999; Ma et al., 2000), lacrimal gland (Moore et al., 2000), sweat gland, (Song, Sonawane & Verkman, 2002), or corneal endothelium (Kuang et al., 2004) where fluid transport is relatively slow.

In the framework of a paracellular model for fluid transport, epithelia which transport at higher rates would require proportionately more regulation via an AQP-linked mechanism. By contrast, from our results above in corneal endothelium (a low-rate layer), this regulatory cycle might affect at most ~20% of the fluid transported. To reiterate, importantly, all our evidence summarized here for corneal endothelial fluid transport and the models we have advanced as a result (Sanchez et al., 2002; Fischbarg, 2003; Fischbarg & Diecke, 2005), including those in this paper (Fig. 12, Fig. 14), are remarkably consistent with the general features of the mechanism for fluid transport proposed by Hill and colleagues (Shachar-Hill & Hill, 2002) in a review covering several other leaky epithelia. In all these cases, the evidence points to a paracellular non-local-osmotic mechanism that depends crucially on the junctions. We attribute junctional coupling to electro-osmosis (Sanchez et al., 2002) and they to mechano-osmosis (Shachar-Hill & Hill, 2002), and that remains to be elucidated. However, this seems minor by comparison with the seemingly extraordinary convergence resulting of the use of the different methodologies noted to arrive at a unified paradigm of paracellular flow.

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