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Electrical parameters and water permeability properties of monolayers formed by T84 cells cultured on permeable supports

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Abstract

T84 is an established cell line expressing an enterocyte phenotype whose permeability properties have been widely explored. Osmotic permeability (P_{OSM}), hydraulic permeability (P_{HYDR}) and transport-associated net water fluxes (J_W-transp), as well as short-circuit current (I_{SC}), transepithelial resistance (R_T), and potential difference (ΔV_T) were measured in T84 monolayers with the following results: P_{OSM} 1.3 ± 0.1 cm.s^{-1} x 10^{-3}; P_{HYDR} 0.27 ± 0.02 cm.s^{-1}; R_T 2426 ± 109 Ω.cm^2, and ΔV_T 1.31 ± 0.38 mV. The effect of 50 µM 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (DCEBIO), a "net Cl^- secretory agent", on T84 cells was also studied. We confirm the reported important increase in I_{SC} induced by DCEBIO which was associated here with a modest secretory ΔJ_W-transp. The present results were compared with those reported using the same experimental approach applied to established cell lines originating from intestinal and renal epithelial cells (Caco-2, LLC-PK1 and RCCD-1). No clear association between P_{HYDR} and R_T could be demonstrated and high P_{HYDR} values were observed in an electrically tight epithelium, supporting the view that a "water leaky" barrier is not necessarily an "electrically leaky" one. Furthermore, the modest secretory ΔJ_W-transp was not consistent with previous results obtained with RCCD-1 cells stimulated with vasopressin (absorptive fluxes) or with T84 cells secreting water under the action of *Escherichia coli* heat stable enterotoxin. We conclude that, while the presence of aquaporins is necessary to dissipate an external osmotic gradient, coupling between water and ion transport cannot be explained by a simple and common underlying mechanism.

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- Aquaporins
- Osmotic and hydrostatic gradients
- T84 cells

Introduction

Epithelial barriers are classified as "tight" or "leaky" according to their electrical conductance (1), which in general decreases with barrier tightness. Two different permeability coefficients of water net fluxes can be measured: osmotic (P_{OSM}) and hydrostatic (P_{HYDR}) (2). Moreover, diffusional water permeability can be measured from unidirectional fluxes using tritiated water. It is accepted that fluxes generated by hydrostatic

pressure gradients (J_W-hydr) move across the intercellular spaces, while the main route for those induced by an external osmotic gradient (J_W-osm) is controversial.

Correlation between electric and water permeability properties is also especially important when a so-called electrogenic ionic transport is observed, resulting in a net salt transfer across the barrier. A net fluid transfer (J_W-transp) is frequently associated with salt movement by a process that is not clearly understood (3,4). These water movements, driven by a salt transport-generated osmotic gradient, could also occur among or across epithelial cells.

The accepted water pathway for transcellular movements is the lipid bilayer itself (5), or specific water channels called aquaporins (6). Alternatively, solute-water cotransport, a controversial mechanism conceptually different from those previously mentioned, has been proposed (7-9). In the present study, water fluxes and electrical parameters were measured in T84 monolayers, a cell line derived from a human colon carcinoma. The original colon epithelium expresses aquaporins in different species, for example humans (10) and rats (11). However, the T84 cell line does not express aquaporins (12) like other established cell lines (10,13). The Na^+/H^+ and Cl^-/HCO_3^- exchangers and the Na^+/HCO_3^- cotransporter have been described in these cells (14). In the present study, minute by minute recordings of the transepithelial net water fluxes (J_W-hydr; J_W-osm or J_W-transp) were associated with the measurement of the transepithelial potential difference (ΔV_T), transepithelial resistance (R_T) and short-circuit current (I_{SC}) under different experimental conditions. The results were compared with those reported for Caco-2, LLC-PK1 and RCCD-1 cells. Furthermore, the effects on water transfer of 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (DCEBIO), recently reported as a "net Cl^- secretory agent" in T84 cells (15), and of bumetanide were also tested and compared with the reported effects of agents that increase active absorption or secretion (12,13,16,17).

Material and Methods

Cell culture

T84 cells, obtained from the American Type Culture Collection (Rockville, MD, USA), were grown to confluent monolayers in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium supplemented with 14 mM $NaHCO_3$, 3.2 mM glutamine, 10 units/ml penicillin-streptomycin, 15 mM HEPES/Na^+, pH 7.4, and 5% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) in a 5% CO_2 atmosphere at 37ºC. For these experiments, cells between passages 58 and 65 were seeded onto Transwell holders (1.10^6 cells/Transwell of 3-µm pore Nuclepore filters, 4.5 cm^2 surface area; Corning-Costar Corp., Cambridge, MA, USA) and cultured for 10 to 12 days.

For the experiments, the T84 cells were bathed on either side with minimum medium containing 1:1 DMEM-Ham's F-12 (Catalog number: 12500-039; Gibco BRL-Life Technologies) and 14 mM $NaHCO_3$ plus 15 mM HEPES/Na^+, pH 7.4, when bubbled with 5% CO_2/95% O_2.

Measurement of water fluxes

In order to perform water flux measurements across the T84 monolayers, the Transwell holders with their bottom covered with the confluent cell layer were directly inserted between two Lucite hemi-chambers so as to define two independent compartments, as previously described (12). One of them (serosal) was open to the atmosphere, while the other (mucosal) was hermetically sealed. A hydrostatic pressure difference (4.5 cm of H_2O) was continuously applied; pressure on the mucosal side is reported relative

to that on the serosal side. The closed chamber was connected with a small diameter polyethylene tube to the net-water-measurement system where the net water flux (J_W) was recorded every minute, as described elsewhere (18). Briefly, the position of a liquid meniscus inside a capillary tube was photoelectrically detected. Displacements to the right or to the left were proportional to the amount of water moving across the tissue layer. The system sensitivity was 50 nl. The data were computed in units of µl min^{-1} cm^{-2}. Mucosa to serosa net movements (absorptive) were considered to be positive fluxes while serosa to mucosa net movements (secretory) were considered to be negative fluxes. The serosal bath was continuously bubbled with the appropriate CO_2/O_2 mixture to maintain pH at 7.4 ± 0.1 (37ºC). J_W-hydr and J_W-osm were the J_W values observed in the presence of a transepithelial hydrostatic pressure gradient or a transepithelial osmotic gradient, respectively. J_W-transp is the remaining J_W when the effects of the external osmotic and hydrostatic gradients are subtracted.

Electrophysiological studies

Transepithelial voltage difference (ΔV_T) and I_{SC} were continuously recorded employing an automatic voltage clamp system (Physiological Instruments, San Diego, CA, USA) and Navycite (ME2AG4) electrodes. Before mounting the preparations in the voltage clamp system, R_T was measured with a Millicell-ERS electric resistance system (Millipore, Bedford, MA, USA). During the experiments, R_T was measured every 90 s from current deflections in response to a 1-mV/second pulse. Regarding ΔV_T, positive or negative values indicate "serosal side positive or negative". Electrophysiological and Jw measurements were carried out simultaneously in the same epithelial layer.

DCEBIO was a generous gift from Prof. Robert J. Bridges, University of Pittsburgh, USA. DMSO was used as solvent to test the effects of DCEBIO (50 µM on the mucosal side, and 50 and 75 µM on the serosal side) on water and ion transport.

Statistical analysis

Data are reported as means ± SEM. Statistical significance was determined using the paired or unpaired *t*-test, and a P value <0.05 was considered to be statistically significant.

Results

Osmotic and hydraulic permeability of T84 cells

Figure 1 shows the J_W values observed when the osmotic gradient across T84 cell monolayers was increased. Gradients were generated by adding mannitol to the serosal bath (the spontaneous water movement, measured in the presence of a 4.5-cm differ-

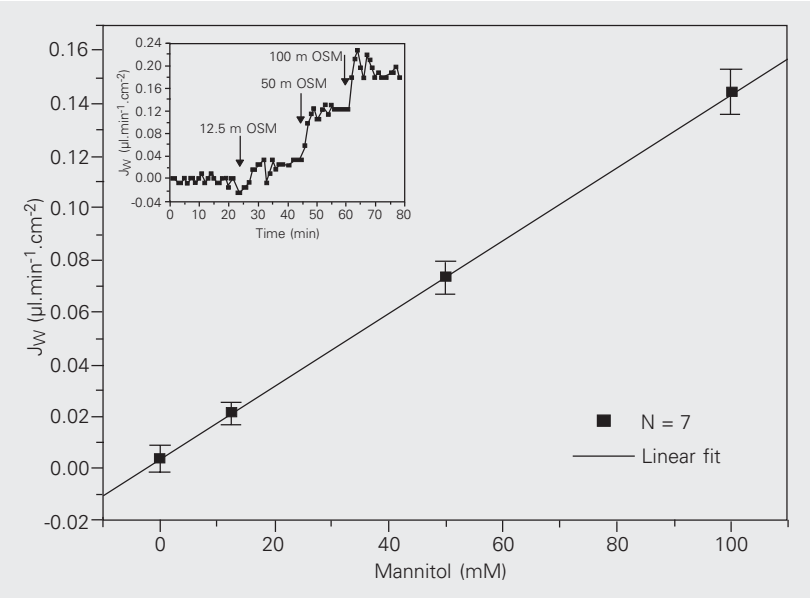

Figure 1. Water flux as a function of osmotic gradients (J_W-osm) applied from the serosal side (12.5, 50 and 100 mM mannitol). Data are reported as means ± SEM (N = 7). The *inset* shows a typical experiment in which water flux did not increase along the time course in the presence of an osmotic gradient. Osmotic permeability (P_{OSM} = 1.30 ± 0.09 x 10^{-3} cm s^{-1}) was calculated from the slope of the corresponding regression curve (R = 0.99 ± 0.04, P < 0.001, *t*-test).

ence of H_2O hydrostatic pressure, was not significantly different from zero in this experimental series). P_{OSM} (Table 1) was calculated from the slope of the regression curve obtained from J_W values and the applied osmotic gradients ($R = 0.99 \pm 0.04$, $P < 0.001$).

Mean J_W values as a function of the applied hydrostatic gradient are presented in Figure 2. P_{HYDR} was calculated from the regression line ($R = 0.99 \pm 0.17$, $P < 0.02$; Table 1). In this case, the spontaneous J_W was a secretory one and reverted to absorptive values under the action of the applied hydrostatic pressure.

Table 1. Osmotic permeability (P_{OSM}), hydraulic permeability (P_{HYDR}), transepithelial resistance (R_T), and potential difference (ΔV_T) observed in different epithelial cell lines cultured on permeable supports.

	Caco-2[a]	RCCD-1[b]	T84[c]	LLC-PK1[d]	Transfected LLC-PK1[d]
P_{OSM} (x10^{-3} cm.s^{-1})	35±6	5.0±0.4	1.3±0.1	7.4±4.8	49.9±8.2
P_{HYDR} (cm.s^{-1})	2.7±0.3	1.0±0.1	0.27±0.02	0.20±0.03	0.25±0.04
R_T (ohm.cm^2)	160±15	2390±140	2426±109	323±13	244±20
ΔV_T (mV)	0.28±0.03	10.5±1.2	1.31±0.38	ND	ND

[a]Parisi et al. (16); [b]Chara O, Parisi M and Capurro C (unpublished results); [c]the present study; [d]Toriano et al. (12). ND = not determined.

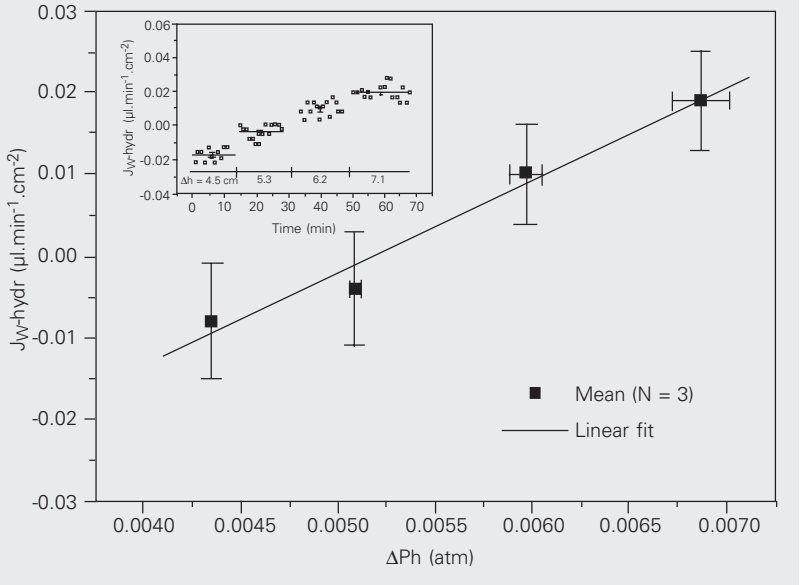

Figure 2. Hydraulic water flux (J_W-hydr) across T84 monolayers as a function of different hydrostatic gradients (ΔPh, atmospheres, N = 3). Gradients were applied from the mucosal side. The hydraulic permeability coefficient (P_{HYDR} = 0.27 ± 0.02 cm.s^{-1}) was calculated from the slope of the corresponding regression curve ($R = 0.99 \pm 0.17$, $P < 0.02$, *t*-test). The *inset* shows that hydrostatic flux did not increase along the time course in the presence of a hydrostatic gradient.

Effects of DCEBIO on electrical parameters and water movements in T84 cells

It has been reported that DCEBIO induces an important secretory Cl^- response in T84 cells, associated with a significant increase in I_{SC} as well (15). We explored the possible effects of DCEBIO on water transfer and also tested the effects of bumetanide, an inhibitor of Cl^- secretion, on this cell line (17).

DMSO was employed as a solvent for both DCEBIO and bumetanide. It was added in a similar concentration to both sides of the cells and did not induce significant changes in J_W, I_{SC}, R_T or ΔV_T. The addition of DCEBIO (50 µM in both mucosal and serosal baths) was followed by a secretory response (ΔJ_W-transp) together with the expected increase in both I_{SC} and ΔV_T (Table 2), effects that were reversed by 10 µM bumetanide. Figure 3 illustrates the results obtained when the concentration of DCEBIO increased on the serosal side after the initial stimulation of both sides. Finally, 10 µM bumetanide was added to the serosal bath.

Discussion

In the present study, we characterized the electrical parameters and water permeability properties of monolayers formed by T84 cells in culture. Low osmotic permeability was observed in this high resistance epithelium, together with rather high P_{HYDR} values. An important difference in potential was associated with these properties, as previously reported (Table 1). We also report that the important increase in short circuit current induced by 50 µm DCEBIO was associated with secretory J_W while both responses were inhibited by 10 µM bumetanide (Table 2).

Comparison of passive electrical and water permeability properties in different epithelial cell lines: the role of aquaporins

The results obtained with T84 cells correlated with data previously reported for different epithelial cell lines cultured on permeable supports. All experiments were performed in our laboratory under similar experimental conditions, permitting comparison in a well-controlled manner. Figure 4 presents P_{HYDR} values plotted against R_T for Caco-2, RCCD-1, T84, LLC-PK1, and LLC-PK1 cells transfected with AQP-2 (Table 1). It can be seen that high resistance values (about 2380 $\Omega.cm^2$) were associated with also rather high P_{HYDR} values (about 1 cm s^{-1}) in RCCD-1 cells. On the other hand, in LLC-PK1 cells much higher conductances were associated with lower P_{HYDR} values. For both P_{HYDR} and R_T, the paracellular route has been previously reported to be the most important one (2,19).

When P_{OSM} and R_T were compared (Figure 5, Table 1), no clear correlation was observed. Values for LLC-PK1 cells transfected with AQP-2 clearly showed that an important increase in P_{OSM} can be associated with no significant changes in R_T.

Finally, no statistically significant correlation between P_{HYDR} and P_{OSM} was observed in non-transfected cells. Furthermore, it should be pointed out that after aquaporin transfection in LLC-PK1 cells, there was a huge increase in P_{OSM} values with no changes in P_{HYDR} (Table 1). Previous studies have demonstrated that T84, LLC-PK1 and RCCD-1 cell lines in culture lose aquaporin expression (13,17,20). Several straightforward interpretations can be proposed on the basis of these observations and of previous and present results: 1) P_{HYDR} is a parameter that represents water transfer across the paracellular pathway. Nevertheless, rather high P_{HYDR} values can be observed in electrically tight epithelia (T84, RCCD-1), confirming that a "water leaky" barrier is not necessarily

Table 2. Effects of 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (DCEBIO, 50 µM both in mucosal and serosal baths) and bumetanide (10 µM) on T84 cell monolayers.

Experimental - basal data	DCEBIO	+ Bumetanide
ΔJ_W-transp (x10^{-3} µl.min^{-1}.cm^{-2})	-13.1 ± 5.2*	18 ± 1.5$^+$
ΔI_{SC} (µA.cm^2)	67.8 ± 22.2**	-47.5 ± 22.5$^+$
ΔV_T (mV)	12.0 ± 2.3*	-7.0 ± 3.7$^+$

Data are reported as means ± SEM (N = 4). ΔJ_W-transp = transport-associated net water fluxes; ΔI_{SC} = short-circuit current variation; ΔV_T = transepithelial potential difference.
*P < 0.001 for J_W-transp and ΔV_T (DCEBIO) against J_W-transp and ΔV_T (basal).
**P < 0.02 for I_{SC} (DCEBIO) against I_{SC} (basal). $^+$P < 0.05 *vs* DCEBIO values (*t*-test).

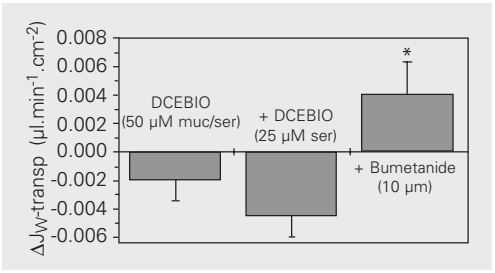

Figure 3. 5,6-Dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (DCEBIO)-induced variations in transport-associated fluxes (ΔJ_W-transp) followed by an additional DCEBIO stimulation (50 µM on the mucosal (muc) and serosal (ser) sides, followed by 25 µM on the serosal side). Finally, bumetanide (10 µM, serosal side) reversed the secretory effect of DCEBIO (mean ± SEM, N = 4). J_W was measured until stable values were achieved. Values were calculated (subtracting basal fluxes) in the absence of osmotic or chemical transepithelial gradients. *P < 0.02 compared to other groups (Student *t*-test).

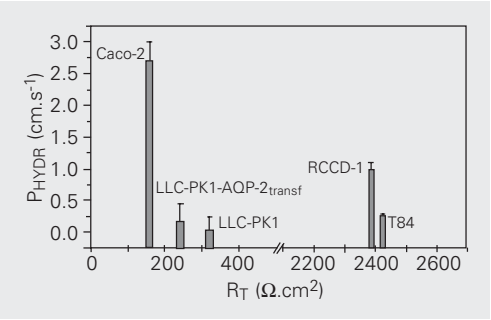

Figure 4. Hydraulic permeability coefficients (P_{HYDR}) and transepithelial resistance (R_T) for different cell lines. Caco-2 = Parisi et al. (16); RCCD-1 = Chara O, Parisi M and Capurro C (unpublished results); T84 = the present study; LLC-PK1 and transfected LLC-PK1 = Toriano et al. (12).

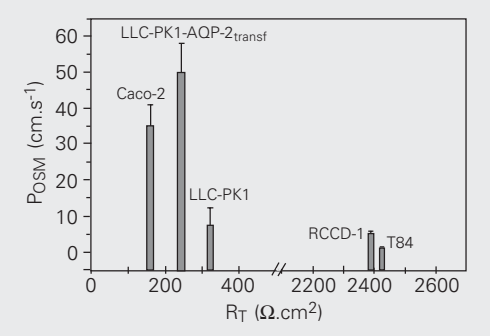

Figure 5. Osmotic permeability coefficients (P_{OSM}) and transepithelial resistance (R_T) for different cell lines. Caco-2 = Parisi et al. (16); RCCD-1 = Chara O, Parisi M and Capurro C (unpublished results); T84 = the present study; LLC-PK1 and transfected LLC-PK1 = Toriano et al. (12).

an "electrically leaky" one (21); 2) in the tested native cells (not expressing aquaporins) no clear correlation was observed between P_{OSM} and R_T. A relatively high P_{OSM} value was observed in the absence of aquaporins in a very leaky epithelium (Caco-2), and 3) after aquaporin transfection, P_{OSM} strongly dissociated from P_{HYDR}, probably because water pathway becomes mainly a transcellular process.

Table 3. I_{SC} and J_W-transp values under the effect of antidiuretic hormone (ADH), *Escherichia coli* heat stable enterotoxin (STa) and 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one (DCEBIO).

	RCCD-1 stimulated with ADH (N = 6)[a]	T84 stimulated with STa (N = 8)[b]	T84 stimulated with DCEBIO (N = 4)[c]
Experimental ΔJ_W-transp ($x10^{-3}$ µl.min^{-1}.cm^{-2})	160 ± 20	-180 ± 40	-13.1 ± 5.2
ΔI_{SC} (µA.cm^{-2})	2.24 ± 0.44	16.9 ± 4.32	67.8 ± 22.2
Theoretical ΔJ_W-transp ($x10^{-3}$ µl.min^{-1}.cm^{-2})	9.3 ± 1.8	-70.1 ± 17.9	-281.3 ± 109.3

STa concentration: 0.25 µM; ADH concentration: 10 mM; DCEBIO: 50 µM. Theoretical values were calculated assuming isotonic flux derived from the electrogenic transport, measured by I_{SC} (see the text). [a]Capurro et al. (13); [b]Toriano et al. (17); [c]The present study. For abbreviations, see legend to Table 2.

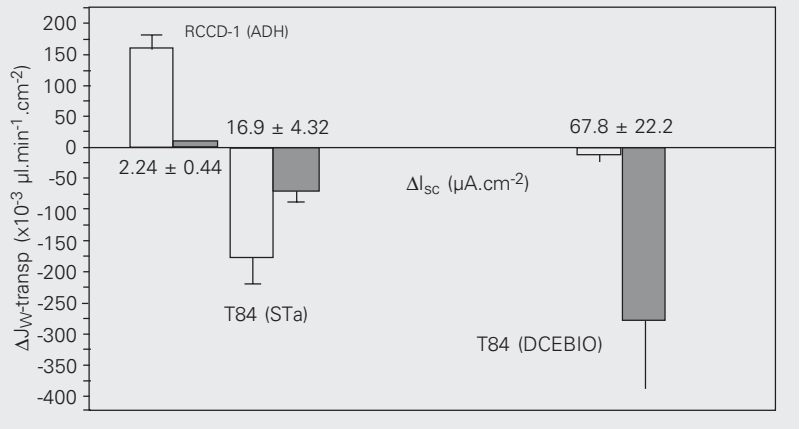

Figure 6. Net water flux (ΔJ_W-transp) and short-circuit current variation (ΔI_{SC}) for different cell lines. The observed ΔJ_W-transp values (open columns) were compared with the theoretical values (filled columns) assuming isotonic transport (see text). The data presented correspond to the experimental ΔI_{SC} values. Both parameters (ΔJ_W-transp and ΔI_{SC}) were measured simultaneously in the same monolayer. RCCD-1 (ADH), Capurro et al. (13); T84 (STa), Toriano et al. (17); T84 (DCEBIO), the present study. ADH = antidiuretic hormone; Sta = *Escherichia coli* heat stable enterotoxin; DCEBIO = 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one.

Water and ion coupling during absorption and secretion

The evaluation of the correlation of I_{SC}, a parameter frequently employed to evaluate ionic transport in epithelial barriers, and the associated increases in J_W-transp is more complex. Three experimental situations were evaluated in previous work from our laboratory and in the present study: the effects of ADH on RCCD-1 (13), the effects of *Escherichia coli* heat stable enterotoxin on T84 (17), and the effects of DCEBIO on this last cell line reported in this paper (Table 3). Figure 6 shows the reported ΔJ_W-transp as a function of the simultaneously measured ΔI_{SC}. The observed ΔJ_W-transp values (open columns) were compared with theoretical values (filled columns), which were calculated assuming that: 1) the electrogenic transport, measured by the I_{SC}, represents the total ionic net transport across the barrier in a pure absorptive or secretory process, and 2) this ionic movement is coupled to an isotonic fluid transfer. In the case of RCCD-1 + ADH, a modest increase in I_{SC} was accompanied by an important absorptive J_W-transp, higher than that predicted if the previously described conditions are accepted. Furthermore, when the effects of *Escherichia coli* heat stable enterotoxin on T84 were analyzed, the observed increase in I_{SC} was coupled to a rather important secretory ΔJ_W-transp. In fact, to accept isotonic movements, a non-electrogenic ionic transport must be postulated in both cases (17).

A completely different situation was observed in the DCEBIO-T84 experiments reported here. An important increase in I_{SC} was associated with a modest ΔJ_W-transp. If we assume that the fluid transfer is isotonic and that the I_{SC} is the result of electrogenic Cl^- secretion, we can expect an induced ΔJ_W-transp of 0.281 µl min^{-1} cm^{-2}. The observed ΔJ_W was 20 times lower (Table 3, Figure 6).

These results can be explained if the observed increments in I_{SC} correspond to the

combination of Na^+ and Cl^- DCEBIO-induced currents. If the increases in Na^+ and Cl^- currents were in opposite directions (mucosa to serosa versus serosa to mucosa fluxes), current values would add up but the associated water movements would be canceled, at least partially. In this context, the Jw-transp calculated from the I_{SC} (accepting isotonic movement) would be lower than that observed. On the other hand, if the increases in Na^+ and Cl^- currents were in the same direction, the result would be that these ionic currents would subtract their values while water fluxes would be added.

Now the Jw-transp calculated from the I_{SC} (accepting isotonic movement) would be higher than that observed. If pure Na^+ or Cl^- increases were detected, appropriate counterions should assure electroneutrality under open circuit conditions. We conclude that the associated water fluxes cannot be accurately determined from I_{SC} measurements.

Water pathways in epithelial barriers

Net water and ionic fluxes can move across epithelial barriers through either transcellular or paracellular routes (2,22,23; for reviews, see Refs. 19,24). Our results indicate that in an epithelium showing important P_{HYDR} values (Caco-2), and in the absence of aquaporins, the paracellular route could be a significant pathway for the osmotic flux. Conversely, in an epithelium presenting lower P_{HYDR} values (LLC-PK1), the presence of aquaporins was crucial to dissipate the external osmotic gradient (25). This last condition would be similar to that described for ADH target epithelia (12).

The route for the "transport-associated" water movement is a highly controversial topic (4), especially regarding secretory processes (26). The "standing gradient model" (27) postulates that the intercellular spaces play a central role in the so-called "isotonic transport-associated water movement". Nevertheless, it has been reported that there is no significant fluid flow through the tight junction of MDCK cells, even when fluid absorption is accelerated by the imposition of an external osmotic gradient (22). Discovery of aquaporin expression in different epithelial cells and particularly in leaky epithelia such as the proximal renal tubule has emphasized the role of transcellular channel-mediated water transport. However, a report from our laboratory demonstrated that, in the case of RCCD-1 cells, an ADH-induced absorptive J_W-transp could develop in the absence of aquaporins (13).

An additional pathway and mechanism for water transport was proposed (i.e., Na^+/glucose cotransporter, SGLT), which would consist of a cotransport of water and solute with a strict stoichiometry of two Na^+, one glucose and ~220-250 water molecules (9,28). This mechanism was also proposed to explain coupling of water and ions in secretory processes. Although this hypothesis has generated strong controversy (29), the authors consider that the local osmotic gradient generated by the solute transfer can explain water transfer (30). Alternatively, it has been recently proposed that electro-osmosis can play a central role in water and ion coupling during active transport in a leaky epithelium (31). However, it should be pointed out that, although results obtained with epithelial cell monolayers represent useful experimental models, they cannot always be extrapolated to natural epithelia.

We conclude that J_W-transp is not always the result of a simple "secondary effect" due to the generation of an osmotic gradient anywhere inside the epithelial barrier. The role of water-solute cotransport in the phenomena described here remains an open question.

Acknowledgments

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